Universidade de Aveiro Departamento de Biologia
2010

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Caracterização do perfil ontogénico da expressão da lipocalina 2

Characterization of lipocalin 2 ontogenic expression profile

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Professor Doutor João Carlos Cruz Sousa, Escola de Ciências da Saúde da Universidade do Minho, e co-orientação da Professora Doutora Odete Abreu Beirão da Cruz e Silva, Professora Auxiliar da Secção Autónomas das Ciências da Saúde da Universidade de Aveiro.

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Agradecimentos

Ao João Sousa pela oportunidade profissional de trabalhar no seu grupo, orientação e conhecimentos partilhados.

À Professora Odete Silva por ter aceite ser minha co-orientadora nesta importante etapa académica.

À Fernanda pela convivência diária, amizade, paciência, por tudo o que me ensinou e pelo apoio prestado neste trabalho.

A todos os NeRD, pela ajuda, amizade e excelente ambiente de trabalho.

A todo o ICVS.

À minha família, principalmente aos meus pais e irmãos, que acreditam em mim, me compreendem e me apoiam incondicionalmente.

À Maria pelo afecto, encorajamento e apoio incondicional dado durante este ano e meio de trabalho.

Palavras-chave Metabolismo do ferro, lipocalina 2, inflamação, lipopolissacarídeo, imunohistoquimica.

Resumo

O ferro é essencial para processos metabólicos fundamentais. Dada a sua natureza reactiva, a homeostasia do ferro é um processo altamente regulado em mamíferos. Um desequilíbrio no metabolismo do ferro tem sido associado a patologias como anemia, hemocromatose e doença de Alzheimer. Ferritina, hepcidina e transferrina são algumas das principais proteínas envolvidas na manutenção dos níveis de ferro no organismo. A Lipocalina 2 (LCN2) é uma proteína de 25 kDa pertencente à família das lipocalinas que tem sido descrita como participante em vários eventos biológicos, tais como diferenciação do rim, protecção de falha renal, apoptose, sobrevivência celular e resposta de fase aguda no fígado, baço, sangue e alguns tecidos epiteliais. Muito relevante é o facto de a LCN2 ser capaz de se ligar a sideróforos bacterianos carregados com ferro. Durante infecções bacterianas, a LCN2 compete com as bactérias pelo ferro disponível, limitando assim a proliferação bacteriana. Este efeito bacteriostático enaltece o papel de LCN2 na resposta imune inata. Dada a capacidade de LCN2 para se ligar a sideróforos carregados com ferro e o facto de existirem células que conseguem capturar este complexo, a LCN2 tem sido proposta como proteína central num putativo mecanismo de entrega de ferro alternativo ao da transferrina. De maneira a compreender melhor o papel de LCN2 no metabolismo do ferro bem como na resposta imune inata, caracterizamos por análise immunohistoquimica o padrão de expressão da proteína em ratinho, do desenvolvimento fetal até um ano de idade, em condições controlo/fisiológicas (injecção com salino) e após estímulo inflamatório agudo (injecção com LPS). A expressão de LCN2 durante o desenvolvimento embrionário foi predominante no fígado e rim. O sinal da proteína persistiu em ambos os órgãos na primeira semana de vida de animais controlo, sugerindo uma ligação da proteína a processos de diferenciação e/ou desenvolvimento. No período pós natal, nos animais injectados com salino a imunoreactividade de LCN2 foi detectada no baço, timo e rim. A injecção com LPS induziu expressão de LCN2 no baço, rim, fígado e barreiras do cérebro. Os dados aqui apresentados indicam que a presença e o perfil de distribuição de LCN2 em alguns tecidos de ratinho são dependentes da idade e estado fisiológico.

Keywords Iron metabolism, lipocalin 2, inflammation, lipopolysaccharide, immunohistochemistry.

Abstract

Iron is essential for key metabolic mechanisms. Given its reactive nature, iron homeostasis is a highly regulated process in mammals. Iron imbalance as been associated to pathologies such as anemia, hemochromatosis and Alzheimer's disease. Ferritin, hepcidin and transferrin are some of the key proteins involved in the maintenance of organism iron levels. Lipocalin 2 (LCN2) is a 25 kDa protein from the lipocalin family that has been reported to participate in a broad range of biological events, such as kidney differentiation, protection from renal failure, apoptosis, cell survival and acute phase response in the liver, spleen, blood cells and some epithelial tissues. Importantly, LCN2 is capable of binding to bacterial siderophores loaded with iron. During bacterial infection scenarios, LCN2 competes with bacteria for iron resources, thus limiting bacterial proliferation. This bacteriostatic effect highlights the role of LCN2 in the innate immune system response. Given the ability of LCN2 to bind iron loaded siderophores and the finding that cells can uptake this complex, LCN2 has been proposed as a central participant in a putative transferrin-alternative pathway for iron delivery. In order to further understand the role of LCN2 in iron metabolism as well in the innate immune system response, we characterized through immunohistochemical analysis the protein expression pattern in mice, from fetal development to one year of age, both in control/physiological conditions (saline injection) and after an acute inflammatory stimulus (LPS injection). LCN2 expression during embryonic development was predominant in the liver and kidney. The protein signal persisted in both organs in the first week of life of control animals, thus suggesting that the protein may be linked to differentiation and/or development processes. In control post-natal animals LCN2 immunoreactivity was detected in the spleen, thymus and kidney. LPS injection induced increased LCN2 expression in the spleen, kidney, liver and barriers of the brain. The present data indicates that LCN2 presence and distribution pattern in some mice tissues is dependent of age and physiological state.

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24p3R, lipocalin 2 receptor

AKI, acute kidney injury

BIM, Bcl-2-interacting mediator of cell death

BMP6, bone morphogenetic protein 6

BMP/SMAD, bone morphogenetic protein / son of mother against decapentaplegic

CP, choroid plexus

CD, collecting ducts

CSF, cerebrospinal fluid

DCYTB, duodenal cytochrome b

DT, distal tubules

DMT1, divalent metal transporter 1 protein

FPN, ferroportin

GFR, glomerular filtration rate

HCP1, heme carrier protein 1

HFE, hemochromatosis

Hox1, heme oxygenase 1

HSC, hematopoietic stem cell

IRE, iron responsive element

IRP, iron regulatory protein

IL-1, interleukin-1

IL-1α, interleukin-1 alpha

IL-1β, interleukin-1 beta

IL-6, interleukin-6

LCN2, lipocalin 2

LPS, lipopolysaccharide

MM, metanephric mesenchyme

NZ, nephrogenic zone

NTBI, non-transferrin-bound iron

PT, proximal tubules

RBC, red blood cells

RBF, renal blood flow

TNF-α, tumor necrosis factor alpha

Tf, transferrin

TfR1, transferrin receptor 1

TfR2, Transferrin receptor 2

TLR, tool like receptor

TLR4, tool like receptor 4

UB, ureteric bud

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Introduction

1.1. Iron metabolism in mammals: an overview

Iron regulation in higher eukaryotes has been intensively studied and characterized. This trace element is required for fundamental biological processes that ensure cellular survival and viability (Hentze et al. 2004), with erythropoiesis being the most iron consuming process known in mammals (Chua et al. 2007). Due to its transition metal nature, iron is easily used in the organism major biochemical redox reactions (Nelson 1999). However this propriety represents a potential hazard for the individual, since iron circulating in the free form can induce "Fenton-type" redox reactions that can create highly reactive radicals that might, in last instance, damage cell genetic and protein content (Hentze et al. 2004). This is the case of iron overload disorders, such as hemochromatosis, that favor the formation of the referred hydroxyl radicals (Dunn et al. 2007). On the other hand, iron shortage is a hallmark feature of anemia (McLean et al. 2009). Furthermore, systemic iron imbalance has also been reported as a contributor for the development of neurodegenerative disorders such as Parkinson´s disease (Mandel et al. 2007). Both iron excess and deficiency can be harmful for the individual and can result in pathological disorders, hence the necessity of a close control of this trace element levels (Crichton et al. 2002). In order to cope with this issue, higher organisms have evolved complex mechanisms that promote iron homeostasis trough uptake, storage and utilization, all of these highly regulated processes (Nelson 1999).

1.1.1. Iron uptake, circulation, recycling and storage

A global overview of the iron cycle in the healthy adult human body is depicted in Figure 1. A healthy human male body contains about 3,5 grams of iron (Muñoz et al. 2009). Dietary iron absorption accounts only for a small percentage of the body iron needs. The dietetic uptake occurs at the apical membrane of intestinal mucosa cells (enterocytes), with the absorption rate being dependent on body iron needs (Chua et al. 2007). Two different forms of iron are found to be captured by enterocytes. The majority of the captured iron (90%) is in an oxidized Fe^{3+} form (non-heme iron). The remaining iron (10%) that is captured is associated with heme protein (heme iron).

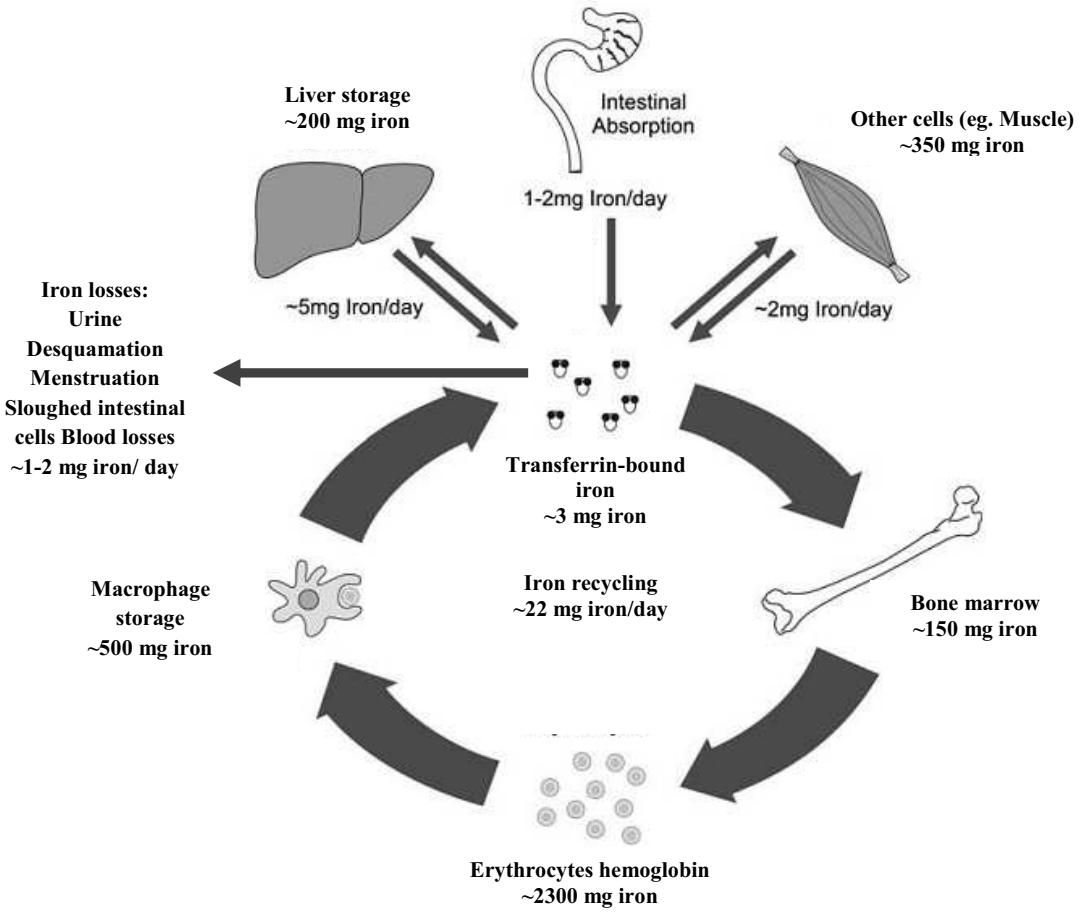

Figure 1 Systemic iron distribution. Dietary iron absorption occurs at the intestine and accounts for a small proportion of the organism iron needs. Iron that circulates in the plasma is bound to transferrin. Most of this available iron is used in erythrocytes formation which occurs in the bone marrow. Indeed, erythropoiesis is the most iron consuming process of the organism, with the majority of the body iron being present in the erythrocytes hemoglobin. Senescent or damaged erythrocytes are phagocyted by macrophages of the reticuloendothelial system which can either store or release the recycled iron into circulation. The quantity of recycled iron that is released by these macrophages is similar to the iron that is consumed in erythropoiesis. Besides macrophages, liver hepatocytes also store iron, with the metal being retrieved from those cells according to the body needs. Iron is also required for the normal functioning of diverse tissues such as muscle fibers. Residual iron losses occur through various processes including skin desquamation, sloughed intestinal cells and blood losses. Adapted from Anderson et al. (2007).

The absorption of these iron forms by the intestinal enterocytes occurs through different mechanisms (Figure 2). Before cellular uptake, non-heme iron is reduced from Fe^{3+} to Fe^{2+} by duodenal cytochrome b (DCYTB) (McKie et al. 2001). Reduced iron then enters the cell through the divalent metal transporter 1 protein (DMT1) (Figure 2). On the other hand, heme iron is likely captured by a putative heme carrier protein 1 (HCP1) and

once within the cell, it is believed that iron is dissociated from the heme protein into the ferrous form (Fe^{2+}) mainly by heme oxygenase 1 (Hox1) (Kikuchi et al. 2005) (Figure 2). This iron presumably shares the same destinations as the absorbed non-heme iron (Muñoz et al. 2009). Once inside the mucosa cell, iron can remain in a labile iron pool (which is ready for immediate utilization by the cell), be stored within ferritin or exported into blood circulation throughout the only known iron exporter, ferroportin (FPN) (Breuer et al. 2008; Chua et al. 2007; Kakhlon 2002; Muñoz et al. 2009).

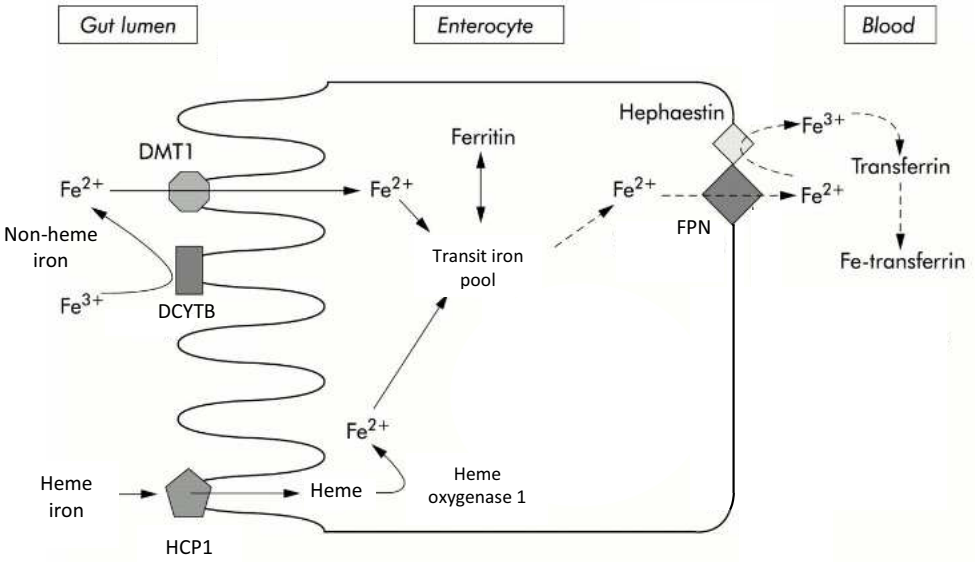

Figure 2 Iron absorption in the enterocyte. Heme iron constitutes a minority of the absorbed iron by the intestinal tract. This iron likely enters enterocytes through heme carrier protein 1 (HCP1) and once within the cell it is released by heme oxygenase 1 (Hox1) in the form of Fe^{2+}. This iron then enters in the transit (labile) iron pool. Non-heme iron is reduced by duodenal cytochrome b (DCYTB) to then enter the cell through DMT1.This iron also joins the transit iron pool. From there iron can either be stored as ferritin or exported to the bloodstream by ferroportin (FPN). Iron is then oxidized by hephaestin to afterwards bind to transferrin, the metal main transporter. All abbreviations can be found on page 7. Adapted from Trinder et al. (2002).

Just before entering circulation, iron is oxidized by the ferroxidase hephaestin (Vulpe et al. 1999). Fe^{3+} then rapidly binds with its main transporter, transferrin. Transferrin is the chief protein responsible for the transport and delivery of iron into the majority of cells (Gomme et al. 2005). The protein has two binding sites for the metal. Therefore, plasmatic transferrin can be apo-transferrin (no iron bound to the protein), monoferric transferrin and diferric transferrin. The protein is then recognized by transferrin receptor 1 (TfR1), a protein present in the cell membrane of most of the organism cells,

which mediates the endocytosis of the transferrin-iron complex. TfR1 has higher affinity for the diferric form of transferrin (Huebers and Finch 1987). Once engulfed, vesicle pH is acidified by injection of H^+ by proton bombs, which in turn promotes the dissociation of iron from transferrin. Afterwards iron leaves the endosome *via* DMT1 transporter into the cytoplasm to be used in diverse cellular processes. Transferrin and TfR1 are then recycled to cell surface for posterior usage (Chua et al. 2007; Crichton et al. 2002; Hentze et al. 2004). Most organism cells uptake transferrin loaded iron through this TfR1 mediated endocytosis mechanism (Hentze et al. 2010).Another transferrin receptor, homologous to TfR1, was first described by Kawabata et al. (1999). Transferrin receptor 2 (TfR2) is mainly expressed in the liver hepatic cells and although it binds transferrin with a lower affinity than TfR1, the cellular uptake mechanism of the transferrin-iron complex trough this receptor is similar to the one described for TfR1. Despite the functional and structural homology between the receptors, in TfR1 absence scenarios, TfR2 is not capable of ensuring basal cell iron uptake needs (Chua et al, 2007).

The levels of dietary iron absorption are identical to the losses of the metal that occurs through events such as skin desquamation, menstruation and sloughing of intestinal mucosal cells (Figure 1). Hence, iron intake by the intestine is not sufficient to ensure the full iron needs of the organism. Indeed, most of the iron required for the regular functioning of the organism is obtained through recycling of senescent erythrocytes. In a healthy human, the majority of the body iron (65-70%) is contained in the red blood cells (RBC) hemoglobin (Anderson et al. 2007). The generation of RBC, a process termed erythropoiesis that normally occurs in the bone marrow, is the most iron consuming phenomena in the organism. Most of the iron required to ensure physiological erythropoiesis is resultant from recycling of the metal from dying or damaged erythrocytes by macrophages of the reticuloendothelial system. Once inside the macrophage, iron is dissociated from hemoglobin and similarly to what happens in enterocytes, it can either be stored in the cell as ferritin, remain in the labile iron pool or exported trough FPN into circulation where it binds to transferrin (Muñoz et al. 2009). A smaller proportion of the body iron (10-15%) is contained in proteins such as myoglobin, NADP dehydrogenase and ferredoxins. The remaining iron (20%) is stored mainly in the reticuloendothelial system macrophages and in the liver hepatocytes. The majority of this stored iron is ferritin bound (Anderson et al. 2007; Muñoz et al. 2009; Nadadur et al. 2008).

Regulation of body iron levels involves diverse organs and tissues (Figure 1). Kidney participation in iron metabolism processes has been gathering increasing interest, with a growing body of evidence now underlining the importance of the organ in iron homeostasis. Accordingly to Smith and Thévenod (2009), approximately 99% of the iron that is filtered in the glomeruli is later reabsorbed in the nephron. The metal uptake appears to occur in proximal tubules (PT) and distal segments of the nephron (Wareing et al. 2000). Concordantly, some of the key iron related proteins have been found to be expressed in the organ.

One of those proteins is DMT1, the main iron importer in the duodenum, which is present in the PT and collecting ducts of rodents (Canonne-Hergaux and Gros 2002; Ferguson et al. 2001; Veuthey et al. 2008). Interestingly, the intra-cellular location of DTM1 in the PT appears to differ between rat and mice, with the protein being present in late endosomes and lysosomes in rat (Abouhamed et al. 2006), while in mice it was detected in the apical side of the cells (Canonne-Hergaux and Gros 2002; Veuthey et al. 2008). Although more work is required to clarify these apparently contradictory observations, the apical localization of DMT1 in PT supports the concept of DMT1 as an iron uptake protein in the kidney (Canonne-Hergaux and Gros 2002; Veuthey et al. 2008). Furthermore, and similar to what was reported in the duodenum (Yeh et al. 2000), the DMT1 expression in the rat kidney is affected by dietary iron intake, with iron-rich diets resulting in lower DMT1 expression, while iron-restricted diets resulted in the opposite effect. Urinary iron levels were also found altered after the dietary manipulation (Wareing et al. 2003). Concomitantly, anemic mice demonstrated an evident increase in DMT1 staining in kidney medulla, with iron deposits being found in some PT (Veuthey et al. 2008). Taken together, these data strongly suggests that DMT1 is involved in iron reabsorption at the kidney level.

Another iron-related protein that has been reported to possibly participate in the recapture of iron from the glomerular filtrate is transferrin. Transferrin, the organism main iron transporter, is small enough to be filtrated by some pores of the glomeruli (Smith and Thévenod 2009). Indeed, residual quantities of the protein are found in the urine of normal subjects (Burne et al. 1999; Sawazaki et al. 1992). Transferrin reabsorption occurs in the apical membrane of PT of the nephron, in a process mediated by the cubilin receptor (Kozyraki et al. 2001). Also, the presence of the transferrin receptor TfR1 in the apical side

of mouse PT suggests that this receptor may mediate the recapture of transferrin from the filtrate, although no concrete evidence supporting this hypothesis exists (Zhang et al. 2007). Given the high affinity of transferrin to iron, practically all of the circulating iron is bound to transferrin. It is then highly likely that the transferrin that is recaptured by the PT is loaded with iron, hence delivering the metal to the PT cells (Smith and Thévenod 2009)(Kozyraki et al. 2001).

While DMT1 and cubilin hypothetically recapture iron from the glomerular filtrate, FPN, the organism main iron exporter that is expressed by various cells types (Veuthey and Roque 2009), was found present in PT cells, suggesting that the protein may export iron into the bloodstream (Veuthey et al. 2008). These authors also observed that under anemic conditions there is a strong increase of FPN signal in the basal membrane of the PT, an evidence that further supports the role of the protein as an iron exporter in the kidney. Interestingly, one of the modulators of FPN expression, hepcidin (Nemeth and Ganz 2009), is also found expressed in the kidney (Veuthey et al. 2008). Whereas in healthy animals hepcidin is strongly expressed in PT cells, during anemia there is an evident reduction of the hepcidin signal in those tubular structures. This pattern of expression suggests that, similar to what happens in other tissues, hepcidin may act as a negative regulator of FPN in the PT cells (De Domenico et al. 2007; Veuthey et al. 2008), thus possibly controlling the export of iron from the nephron into blood circulation.

Taking these data in consideration, it becomes evident that the kidney may have a more important role in iron homeostasis than until recently expected. Alteration in the expression of iron transporting proteins such as DMT1 and FPN in situations where iron demand is higher, such as in anemia and iron-deprived diet clearly suggests that there is a re-uptake of the metal from the glomerular filtrate, with this iron likely being freed back into systemic circulation.

1.1.2. Regulation of iron homeostasis

As referred above, iron imbalance can be hazardous for the organism. Given that mammals do not possess efficient mechanisms for the excretion of the metal, the regulation of the body iron levels must be tightly controlled. This regulation occurs in all steps of iron handling, from intestinal absorption of the metal to its cellular utilization. The homeostasis

of iron takes place both at the cellular and at the systemic levels, with several molecules and mechanisms being responsible for the modulation of key iron related proteins (Hower et al. 2009).

1.1.2.1. Cellular iron metabolism

Mammalian cells require iron for key metabolic processes. Cells can acquire, use, store and export iron, processes that are controlled by various proteins such as DMT1, ferritin and FPN. The modulation of those iron related proteins by the cell can occur at transcriptional, post-transcriptional and post-translational levels.

Regulation of some iron related genes at a transcriptional level can respond not only to cellular iron levels but also to factors such as cytokines, lipopolysaccharide (LPS) and hypoxia. For instance, inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-2 are found to up-regulate the mRNA expression of ferritin in diverse mammal cells (Torti et al. 2002). Likewise, interleukin-6 (IL-6) can regulate hepcidin mRNA synthesis (Lee et al. 2005).

The post-transcriptional regulation of iron metabolism-related genes is considered essential for the maintenance of adequate cellular iron load and is centered in the iron responsive element/ iron regulatory protein (IRE/IRP) system. IRPs bind to IREs, which are hairpin loops present in untranslated regions (UTR) of iron-related proteins mRNAs. The translation outcome of this binding is dependent of the positioning of IRE in either 5′or 3′UTR of the target mRNA. Hence, if IRP binds to the IRE at the 5′-UTR of the target gene, the translation of the gene gets inhibited. On the other hand, the formation of IRP/IRE complex in the 3′-UTR of the gene leads to a higher stability of the target mRNA, resulting in higher gene expression (Muckenthaler et al. 2008). Proteins such as ferritin and FPN possess IRE at the 5′-UTR of their mRNA, while in Tfr1 and DMT1 mRNA the IRE is at 3′-UTR (Chua et al. 2007).

The binding activity of IRP to IRE is dependent of the intracellular iron levels, namely of the labile iron pool status. There are two homologous forms of IRP in the mammalian cells, IRP1 and IRP2, and whereas both proteins bind to IRE, the mechanism by which iron modulates this binding activity is distinct between IRPs. Hence, if cellular iron levels are high, IRP1 complexes with an iron-sulfur cluster, resulting in the protein

inability to bind IRE. Simultaneously, IRP2 is targeted for proteosomal degradation, which leads to lower IRP2 levels in the cell. On the other hand, if iron levels are low, both IRPs are available to bind IREs (for review see Hower et al. (2009)).

Post transcriptional modulation of various iron related proteins by the IRP/IRE system has been demonstrated. In cellular iron depletion scenarios, IRPs binding to 5´-UTR IRE proteins such as ferritin and FPN decreases the translation of both proteins, whereas IRP binding to 3´-UTR IRE of Tfr1 stabilizes the protein transcript. This results in increased iron uptake from the extra cellular medium as well decreased export of the metal. Consequently, iron concentration in those cells increases. Conversely, when cells are iron replete, IRPs lose their IRE binding capability. As a result, ferritin expression increases and the opposite occurs to Tfr1, thus preventing additional entrance of the metal into the cell (Muckenthaler et al. 2008; Viatte et al. 2009). Although a DMT1 isoform is reported to possess an IRE in the 3´UTR of the protein mRNA transcript, there is no concrete evidence that the protein expression is modulated by the IRE/IRP system (Hentze et al. 2010).

It is important to notice that the majority of these molecular mechanisms were found and described in *in vitro* work. However, increasing *in vivo* evidence support the relevance of these mechanisms in cellular iron homeostasis (Ferring-appel et al. 2009; Galy et al. 2008; Gnana-Prakasam et al. 2010; Muckenthaler et al. 2008). It is also noteworthy that, despite the presence of IRPs in renal PT, the role of the IRE/IRP system in the regulation of iron related molecules in the kidney remains unclear (Muckenthaler et al. 2008).

Besides transcriptional and post-transcriptional regulation, some iron related proteins such as TFR2 and FPN were found to be regulated after translation of the respective transcripts (Johnson and Enns 2004; Yeh et al. 2000). Details of FPN post-translational regulation by hepcidin will be described below.

1.1.2.2. Systemic regulation of iron metabolism: The hepcidin model.

As described above, cells can manage their own levels of iron through the regulation of iron related proteins. Adequate iron levels at the systemic level must be ensured by coordinated regulation of iron absorption, storage and recycling. Hence, feedback mechanisms must exist in order to balance those different activities in accordance

to the organism iron needs. Growing evidence suggests that at the axis of such mechanism is hepcidin.

Hepcidin is a liver produced hormone that displays anti-microbial characteristics (Park et al. 2001). The first evidence on the importance of hepcidin in iron metabolism came from work with transgenic mice that lacked the hepcidin gene as a consequence of the knocking-out of a neighbor gene. Those mice presented a hemochromatosis phenotype, with iron deposition occurring in the liver and pancreas (Nicolas et al. 2001). Since then, the understanding on the biology of hepcidin increased greatly and the protein is now considered pivotal in the systemic regulation of iron (Hentze et al. 2010).

Hepcidin acts by binding to the iron exporter FPN which is present in enterocytes, macrophages and hepatocytes, promoting the internalization and degradation of the exporter, which results in the inhibition of iron release from those cells (Figure 3). Consequently, serum iron levels decrease. Hepcidin thus acts as a negative regulator of iron export from the above mentioned cells (Nemeth et al. 2004; Ramey et al. 2010).

Hepcidin expression levels can respond to a variety of factors. Iron overload or inflammatory stimulus results in increased expression of hepcidin and consequent diminution of iron export from enterocytes, macrophages and hepatocytes (Lee et al. 2005; Muñoz et al. 2009; Pigeon et al. 2001) (Figure 3). On the other hand, the expression of hepcidin is found to be down-regulated when the body requires higher amounts of available iron, which is the case in anemic condition, hypoxia and increased erythropoiesis (Frazer et al. 2002; Frazer et al. 2004; Millard et al. 2004) (Figure 3). This group of evidence supports hepcidin as a major regulator of mammalian iron metabolism.

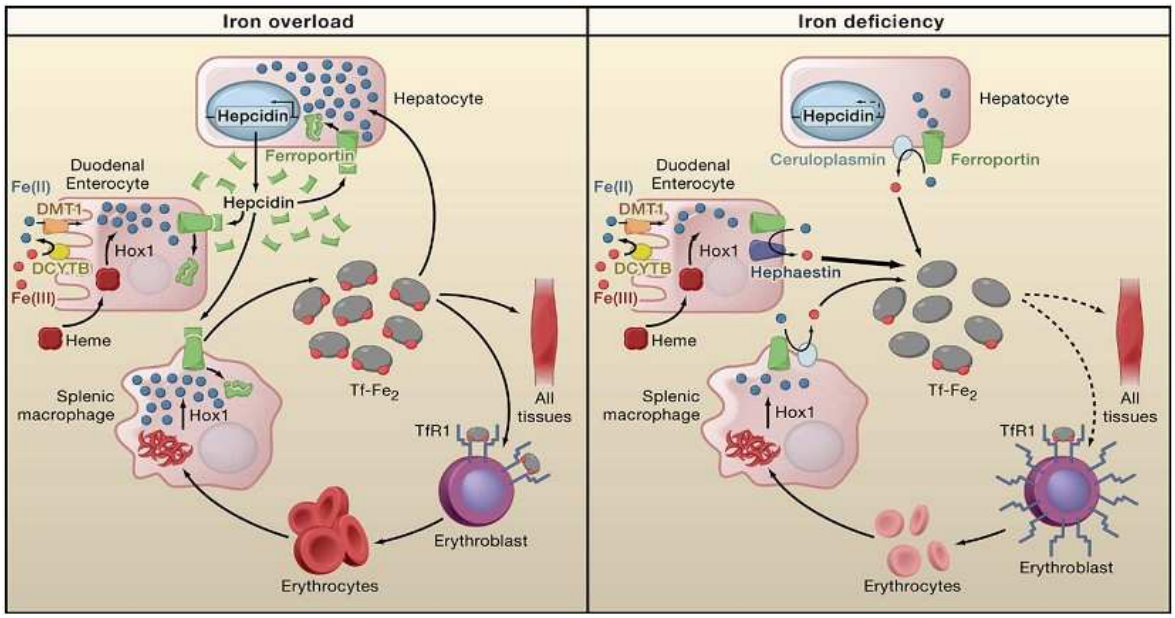

Figure 3 Systemic iron regulation. Regulation of iron influx occurs in all steps of the metal handling, from intestinal absorption to its cellular utilization. Details of those processes are discussed in the text. The maintenance of adequate iron levels at the systemic level is ensured by the liver produced hepcidin. Hepcidin acts through binding to the iron exporter ferroportin, which is present at the surface of enterocytes, splenic macrophages and hepatocytes. This binding promotes the exporter internalization and degradation. Consequently, iron is not exported by those cells. In iron overload scenarios (left), increased hepcidin expression by hepatocytes results in reduced iron export by hepatocytes, macrophages and enterocytes. Conversely, when iron levels are low (right), hepcidin synthesis is decreased, allowing ferroportin to export iron into circulation. All abbreviations can be found on page 7. Reproduced with permission from Hentze et al.(2010).

The adequate expression of hepcidin is essential for the maintenance of the organism iron homeostasis. Hepcidin deregulated expression is associated to some iron related pathologies (Nicolas et al. 2001; Nicolas et al. 2002). As referred above, various factors can influence hepcidin expression and details of the molecular pathways behind such expression modulation have been unveiled (for review, see Viatte and Vaulont (2009) and Hentze et al. (2010)). Thus, hepcidin induction by inflammation was found to be dependent of the activation of the JAK/STAT3 pathway by IL-6 (Wrighting and Andrews 2006). On the other hand, hepcidin expression is decreased by erythroid precursor protein GDF15 (Tanno et al. 2007), although the molecular pathway responsible for this regulation is still unknown. The control of hepcidin expression by systemic iron levels appear to rely on different molecules and mechanisms. For instance, there is evidence that suggest that hemochromatosis (HFE), the principal responsible for the iron overloading disorder hereditary hemochromatosis, promotes hepcidin synthesis in hepatocytes through the

formation of a HFE-TfR2 complex in response to high plasmatic iron levels (Hentze et al. 2010). However, growing evidence suggest that the bone morphogenetic protein / son of mother against decapentaplegic (BMP/SMAD) signaling pathway is the key mechanism of hepcidin modulation by systemic iron. Indeed, bone morphogenetic protein 6 (BMP6), whose expression is iron-dependent (Kautz et al. 2008), was recently reported as being a major regulator of hepcidin expression. Meynard et al. (2009) and Andriopoulos et al. (2009) demonstrated that BMP6 induces hepcidin expression in hepatocytes through the BMP/SMAD pathway, with BMP6-null mice presenting residual hepcidin expression and consequent organ iron accumulation. Although further work is required in order to understand how BMP6 expression is modulated by iron, these novel findings recognize BMP6 as a new master regulator of iron metabolism.

1.2. Lipocalin 2, a new member in the iron metabolism proteins family

In normal conditions, the intake of iron in most of the cells occurs through the previously described transferrin pathway. However, growing evidence suggests the presence of alternative mechanisms for the delivery of the metal into the cells. Studies with transferrin deficient mice (hypotransferrinemic) demonstrated that despite the incidence of anemia and deficiency in the central nervous system development, most of the epithelial organs are normal in this mouse strain (Yang et al. 2002). These animals also accumulate iron in the liver. Together, these facts support the presence of alternative pathways to transferrin-dependent cellular iron uptake. The participants and *modus operandi* of those mechanisms remain largely elusive. At the core of one of those putative mechanisms is lipocalin 2 (LCN2).

The lipocalin family comprises a heterogeneous group of proteins that share tertiary structure and regions of sequence homology. Lipocalins are transporters of small hydrophobic ligands and have been linked to various biological processes (Grzyb et al. 2006). LCN2, also known as 24p3 or neutrophil gelatinase-associated lipocalin (NGAL), is a 25 kDa sized lipocalin that was first reported in human neutrophils (Kjeldsen et al. 1993). Homologous forms of the protein were later confirmed in rodents (Kjeldsen et al. 2000). Key features of LCN2 are its ability to bind iron loaded siderophores (small molecular chelators secreted by bacteria) and its participation in iron trafficking (Devireddy et al.

2005; Goetz et al. 2002; Yang et al. 2002). We will next consider the functions of LCN2 in further detail.

1.2.1. Mechanism of action of LCN2 in iron delivery

The importance of LCN2 in iron related processes was first demonstrated by Yang et al. (2002) that showed that LCN2 promotes the conversion of rat metanephric mesenchyma into epithelia, through cellular iron delivery by a transferrin alternative pathway. It was also apparent that the internalization of either transferrin or LCN2 was dependent of the organ developmental phase, with earlier epithelial progenitors incorporating LCN2 while the further staged epithelial cells internalized mainly transferrin (Yang et al. 2003). Furthermore, the authors also demonstrated in cultured cells that iron delivery by LCN2 regulates iron-responsive genes, such as ferritin and TfR1. Surprisingly, LCN2 knockout mice present normal kidney function, thus suggesting that LCN2 mediated iron delivery is not crucial for the organ development (Berger et al. 2006). Together, these data indicates that at least part of the biological significance of LCN2 is linked to its capacity to bind iron.

LCN2 does not bind to iron directly. Instead, the protein conjugates with small compounds that chelate the metal, such as the bacterial siderophore enterobactin (Goetz et al. 2002). A putative mechanism of LCN2 mediated iron traffic was later proposed by Devireddy et al. (2005). In experiments using modified HeLa cells it was shown that LCN2 can either promote the cellular intake of the metal or it's withdraw from the cellular iron pool depending on the LCN2 iron status. Fundamental to this behavior is the presence of a LCN2 cell surface receptor, 24p3R. Hence, once siderophore iron loaded LCN2 (Holo-LCN2) is recognized by 24p3R, it gets internalized by receptor-mediated endocytosis. The bound iron is afterwards released, leading to an increase in the intra cellular iron concentration. On the other hand, 24p3R can also uptake LCN2 with no iron and siderophore bound to it (Apo-LCN2). After internalization, the protein associates with a putative intra-cellular siderophore which, in turn, is bound to intracellular iron. This complex is then excreted to extracellular space, resulting in a diminished intra-cellular iron concentration (Devireddy et al. 2005).

Questions relative to this model of iron delivery persist. Of special importance is the origin and nature of the putative eukaryotic siderophore. It is well established that bacteria produce siderophores, but the same does not apply to eukaryotic cells as there is no evident proof that eukaryotic cells synthesize siderophores (Richardson 2005; Schmidt-Ott et al. 2007). The biological relevance of this mechanism in physiological conditions such as organ development thus remains unclear, since no bacterial siderophore is present in those circumstances. The isolation of a putative eukaryotic siderophore or homologous compound is then critical for further understanding and validation of this iron delivery pathway.

1.2.2. LCN2 and immunity

In earlier reports LCN2 was found constitutively expressed in tissues that are exposed to external environmental pressures, such as the gut and pulmonary tract (Cowland and Borregaard 1997; Friedl et al. 1999). It was later found that inflammatory stimulus induces acute LCN2 expression in the lung (Sunil et al. 2007), primary cultures of skin epithelia (Cowland et al. 2003), adipocytes (Sommer et al. 2009), macrophages (Flo et al. 2004) and epithelial cells of the choroid plexus (Marques et al. 2008). This acute response to an inflammatory stimulus is even more evident in the liver, suggesting that this organ is the major producer of the protein (Flo et al. 2004) under these conditions. This prompt and ubiquitous response lead researchers to hypothesize that LCN2 could somehow be part of the innate immune response mechanisms. Bacteria require iron for growth and proliferation in an infection setting. To cope with this need, bacteria produce siderophores that harvest iron resources from the host (Neilands 1995). Hence, and due to its high affinity for siderophores, LCN2 competes with the pathogen for iron, thus restraining the bacteria propagation *in vitro* (Goetz et al. 2002). This bacteriostatic effect of LCN2 was later demonstrated *in vivo* by Flo et al. (2004) and Berger et al. (2006); it was shown that LCN2 deficient mice injected with *Escherichia coli* presented higher bacteraemia and bacterial counts in the liver and spleen when compared to wild type animals. The mortality rates due to sepsis were also higher in knockout animals. Hence, LCN2 exerts a role in antibacterial iron-depletion mechanisms of the innate immune system. This effect also provides a plausible explanation for the basal expression of the protein in the respiratory

and intestinal tract. Up-regulation of LCN2 protein has also been found in various inflammatory conditions such as inflammatory bowel disease, appendicitis and diverticulitis (Nielsen et al. 1996).

1.2.3. Other functions of LCN2

1.2.3.1. LCN2 in the healthy and injured kidney

As discussed before, LCN2 appears to participate in kidney development through iron delivery to immature renal epithelia. The role of the protein in the normal functioning of the mature kidney remains, however, unclear. Nevertheless, there is some evidence sustaining that LCN2 may also deliver iron into the healthy kidney. LCN2 is a protein small enough to be easily filtrated by the glomeruli. Despite that, residual levels of LCN2 are found in the urine (Mori et al. 2005; Yang et al. 2003), suggesting that most of the protein is recaptured in the nephron. Indeed, intraperitoneally injected exogenous LCN2 is promptly cleared from the blood by kidney PT cells, with a small fraction of the injected protein being excreted into the urine (Mori et al. 2005). Concomitantly, the putative LCN2 receptor megalin is expressed in the PT cells and megalin deficient mice excrete higher quantities of LCN2 to the urine (Mori et al. 2005), indicating that megalin may indeed mediate the intake of LCN2 in the PT. Expression of 24p3R, another putative LCN2 receptor, has not been described in the kidney. Furthermore, Mori et al. (2005) also reported that shortly after intraperitoneal injection with ^{55}Fe-loaded LCN2, most of the radioactive iron was found concentrated along the apical side of mice kidney PT cells. Whereas this group of data demonstrates that exogenous LCN2 filtrated by the glomeruli is captured by PT and delivers iron into the kidney, there is no concrete evidence that the endogenous protein exerts identical functions in healthy individuals.

Much attention has been given to LCN2 in the context of acute kidney injury (AKI). LCN2 is massively up-regulated in the mouse kidney, serum and urine during ischemia-reperfusion injury (Mori et al. 2005; Schmidt-Ott et al. 2007). Administration of nephrotoxins also induces strong LCN2 expression in the kidney of rodents (Mishra et al. 2004; Mori et al. 2005; Wang et al. 2008). In humans, the up-regulation of LCN2 in the

serum and/or urine following conditions such as kidney transplantation, cardiac surgery, haemolytic uraemic syndrome and sepsis correlates positively with AKI development (Mishra et al. 2005; Mishra et al. 2006; Mori et al. 2005; Trachtman et al. 2006; Wagener et al. 2006; Wheeler et al. 2008). Furthermore, increased LCN2 expression in renal tissue shortly after renal transplantation predicts AKI (Mishra et al. 2006). Concomitantly, in AKI scenarios LCN2 is detected in blood and urine earlier than creatinine, one of the most commonly used biomarkers for kidney damage (Devarajan 2008a). Therefore, LCN2 is regarded as a promising biomarker for early detection of kidney injury, with effective clinical applications in perspective (Devarajan 2008; Di Grande et al. 2009; Mori and Nakao 2007). Some reports also demonstrate that LCN2 is a marker of chronic kidney disease, reflecting the severity of the renal damage (Bolignano et al. 2008; Kuwabara et al. 2009; Mitsnefes et al. 2007).

The biological relevance of LCN2 in the context of renal injury remains relatively unclear. The existing evidence proposes that LCN2 exerts a protective effect in the injured kidney. Indeed, injection of exogenous LCN2 either before or after ischemia-reperfusion injury attenuates the organ damage (Mishra et al. 2004; Mori et al. 2005). Pivotal to this effect appears to be the delivery of iron to the kidney PT by LCN2. Furthermore, LCN2-siderophore-iron complex capture by the PT cells results in higher expression of local Hox1, an enzyme that protects the renal epithelia from diverse damage (Jarmi and Agarwal 2009) and rescue of the epithelial marker N-cadherin (Mori et al. 2005). The up-regulation of Hox1 by LCN2 appears to be crucial to the LCN2 mediated protective effect, with Hox1 inhibition resulting in the neutralization of this LCN2 activity (Mori et al. 2005). It must be noticed that the origin of the LCN2 protein present in the PT after the ischemic injury is probably extra-renal. Indeed, in AKI scenarios the LCN2 mRNA is found up regulated in the lungs and liver, which may secrete the protein into circulation (Devarajan 2010; Schmidt-Ott et al. 2006). Hence, the observed LCN2 signal in the kidney PT is most likely resultant from systemic LCN2 reabsorption of the glomerular filtrate (Mori et al. 2005).

On the other hand, in the AKI setting the renal synthesis of LCN2 mRNA occurs mainly in the distal portion of the nephron, namely in the colleting ducts and loop of henle (Schmidt-Ott et al. 2006). As a significant portion of this locally produced LCN2 appears to be excreted into the urine, it is plausible that LCN2 may exert bacteriostatic functions in

the distal portion of the urinary tract (Mori et al. 2005; Schmidt-Ott et al. 2006; Schmidt-Ott et al. 2007).

In summary, the existing data indicates that LCN2 in the injured kidney may act at two levels. Thus, LCN2 captured from glomerular filtration may promote PT survival, hence attenuating the renal damage. On the other hand, LCN2 synthesized in the distal portion of the organ eventually protects lower urogenital tract from bacterial infections (Schmidt-Ott et al. 2006; Schmidt-Ott et al. 2007).

1.2.3.2. LCN2 in cell survival and apoptosis

Increasing evidence suggests that LCN2 exerts other functions other than iron trafficking and infection modulation. Diverse studies report that LCN2 displays contradicting effects in cell survival modulation. The work of several authors supports LCN2 as an apoptotic facilitator, at least in *in vitro* systems (Devireddy et al. 2005; Lee et al. 2007; Lee et al. 2009; Leng et al. 2008; Miharada et al. 2005; Miharada et al. 2008; Tong et al. 2003). Interestingly, some of these works indicate that the iron transport ability of the protein is linked to this pro-apoptotic behavior. Devireddy et al. (2005) demonstrated that the lipocalin-mediated depletion of intra-cellular iron resulted in the up-regulation of the pro-apoptotic Bcl-2-interacting mediator of cell death (BIM) protein, accompanied by an apoptotic phenotype. Identical conclusions were obtained in work with astrocytes, where the cell death sensitization activity of the lipocalin was abolished by the presence of siderophore-iron complexes. An increased BIM expression after LCN2 treatment was also detected in those cells (Lee et al. 2009).

Apoptosis is a highly regulated process that can be influenced by endogenous and exogenous factors. It is possible that rather from being directly pro-apoptotic, LCN2 may, in certain circumstances, increase the cellular death by modulating the availability of signaling factors (Kehrer 2009). The apoptotic effect of LCN2 can also be adjusted by the organism necessities. Accordingly, some authors propose that LCN2 putative regulation of the hematopoietic process (trough apoptosis and inhibition of cell differentiation) may be dependent of the body needs of certain hematopoietic lineages (Kehrer 2009; Miharada et al. 2005; Miharada et al. 2008).

In contrast with the pro-apoptotic evidence, LCN2 was also described as a survival factor. The findings of Tong et al. (2005) include normal proliferation of A549 cells that were over expressing LCN2 and an increase in the apoptotic effect of celecoxib-related compounds in cells that were silenced for the lipocalin by small-interfering RNA (siRNA). Also, the high expression of LCN2 in various cancers, in normal mammary glands, uterus and testis is concomitant with a survival function (Kehrer 2009).

1.2.3.3. LCN2 in neoplasia

LCN2 is found highly expressed in diverse types of human cancer such as ovarian, breast, colorectal and lung cancer. The biological significance of this expression is not clear, with the protein promoting different effects in different types of cancer. For instance, LCN2 was found to restrain the invasive and metastatic phenotypes in colon cancer cells (Lee et al. 2006). Conversely, LCN2 appears to facilitate the regeneration of the gastrointestinal mucosa through promotion of cellular migration (Playford et al. 2006). Furthermore, LCN2 is linked with the invasive potential of breast cancer (Leng et al. 2009).

1.2.3.4. LCN2 in thermal stress

An unexpected role for LCN2 in thermal protection of the cell was recently described by Roudkenar et al. (2009). In this work, CHO and HEK293T cells that were transfected with lipocalin 2 presented higher proliferative rates after a cold stress, when compared with controls. The number of apoptotic cells and the expression of pro-apoptotic proteins were also lower in the transfected cells. Accordingly, A549 cells that were silenced via siRNA for the LCN2 gene were more prone to apoptosis. An increase in the expression of the protein in the liver, heart and kidney of mice that were subjected to thermal stress was also noticed. Overall, these results suggest a cytoprotective role for LCN2 against cold stress, a function that might be useful for the reduction of cold associated damage in the storing of organs for transplantation (Roudkenar et al. 2009).

1.3. Our work: goals and methodologies

The biological importance of LCN2 is far from fully understood. To our knowledge, important and/or precise functions of the protein in physiological conditions remain unclear. LCN2 expression has been reported in diverse tissues, under various conditions, in varied animals species and strains. Hence, to our knowledge, a coherent and exhaustive description of the ontogenic expression profile of LCN2 in a single biological system in either physiological or inflammatory conditions does not exist.

The aim of this work is to determine the LCN2 expression pattern (mainly by immunohistochemical analysis) in mice during fetal development, early stages of adult growth and in later ages (by analyzing specific organs such as liver, spleen, thymus and brain) under physiological/control (saline injection) and acute inflammatory conditions (24h after LPS administration). With this description, we expect to contribute to the characterization of the role of LCN2 in the innate immune system response and in iron delivery.

2

Experimental Procedures

2.1. Animals and treatments

All animal experiments followed the European Community Council Directive 86/09/EEC guidelines for the care and handling of laboratory animals. The C57BL/6 mice (Charles River, Barcelona, Spain) were maintained in a 12-h light/dark cycle at 22.5 °C and 55% humidity and fed with regular rodent chow (Mucedola, Milano, Italy) and tap water *ad libitum*.

For this study C57BL/6 mice embryos from developmental stages E11,5, E13,5, E15,5, E17,5 and E19,5 were used. Pregnant progenitors were intraperitoneally injected with either control solution (0.9% NaCl) or with 5 μg/g body weight of LPS (Escherichia coli, serotype O26:B6; Sigma, St Louis, USA). We have previously shown that this LPS dosage induces acute inflammatory response in mice (Marques et al. 2008; Marques et al. 2009). Twenty four hours later animals were anesthetized, transcardially perfused with saline and embryos harvested into 4% PFA in PBS. For the analysis of LCN2 expression pattern in post natal animals only male mice were used. The analyzed ages were 3, 7, 16, 21, 30, 60, 120, 365 days. The injections procedure was the same as described above. After anesthetized and transcardially perfused with saline, organ samples (brain, liver, thymus, spleen and kidney) were collected and fixed in 4%PFA in PBS for 5 days. For each condition at least 3 animal samples were used.

2.2. Immunohistochemistry

After fixation, embryos and organ samples were embebed in paraffin and sectioned (4 μm) for immunohistochemistry. All staining steps were performed at room temperature. Briefly, tissue sections were deparaffinized in xylol and ethanol, rehydrated and microwave heated for 20 minutes with citrate buffer (Thermo) for antigen retrieval Samples were washed in PBS and endogenous peroxidase activity was quenched in 0.3% H_2O_2 in PBS for 30 minutes. Samples were rinsed with PBS and blocked with 0,4% bovine serum albumin in 0,3% triton PBS (0,3% PBS-T) for 1 hour. Sections were afterwards incubated with goat anti-mouse LCN2 (R&D Systems, Minneapolis, MN, USA) overnight. Samples were washed with PBS-T and incubated with biotinylated anti-goat secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA) at a 1:200 dilution for an hour and afterwards incubated with avidin-biotin immunoperoxidase reagent (Vectastain Elite

peroxidase ABC kit, Vector Laboratories). Finally, the peroxidase was revealed with 3,3′-diaminobenzidine (DAB; Sigma Immunochemicals, St. Louis, USA) dissolved in Tris-HCl/H_2O_2. Samples were counterstained with hematoxylin and when necessary were stained with periodic acid-Schiff reagent (PAS). Negative controls included incubation with 0,4% BSA in PBS-T without the primary antibody.

Given the high number of samples to be analyzed, we had to repeat this protocol multiple times. To ensure comparable results through all the samples, we tried to ensure identical conditions in each time the protocol was executed.

2.3. Immunofluorescence

For immunofluorescence experiments, a slightly modified protocol was used. No endogenous peroxidase quenching was necessary. A custom made rabbit anti-mouse TTR antibody (Sousa et al. 2007) was used at dilution of 1:1000. We used the following secondary antibodies at a 1:500 dilution: donkey anti-goat Alexa Fluor 488, donkey anti-goat Alexa Fluor 594 and donkey anti-rabbit Alexa Fluor 594 (Molecular Probes, Carlsbad, CA, USA). Sections were mounted with ImmuMount mounting medium (Thermo). In some samples we added DAPI (Thermo) at a 1:2000 dilution, which stains the cell nuclei, to the mounting medium.

2.4. Image acquisition and statistical analysis

The samples were examined under optical (BX61; Olympus, Hamburg, Germany) or confocal (FV1000; Olympus, Hamburg, Germany) microscopes. To quantify the differences in terms of LCN2 expressing cells in the liver of some animals, series of 5 micrographs per animal (n=3 animals per condition, with the exception of some embryo time points/mother injection) covering an area of 0.149 mm^2 each were taken and cells were counted. We did the same for the spleen, with the micrographs (covering an area of 0.0367 mm^2 each) being taken in the red pulp area of the organ. LCN2 positive cell numbers are reported as mean ± s.e. and statistical differences were assessed using standard two tailed Student t test, with differences considered significant at $p<0.05$.

3

Results

In this study we characterize the ontogenic expression profile of LCN2 in mice, under physiological conditions and after an acute inflammatory stimulus. As described in detail in the materials and methods section, embryos and target tissue samples were collected 24 hours after injection of either saline or LPS, fixed in formalin and embedded in paraffin. Tissue sections were then analyzed after LCN2 immunohistochemistry and/or immunofluorescence. A detailed description of the LCN2 expression pattern for each sample analyzed follows. An overview of the expression pattern of the protein can be found in Table 1.

3.1. LCN2 expression pattern in the liver

The expression of LCN2 was first detected in the liver of control animals at the embryonic age 11,5 (E11,5) (Figure 4). As can be observed in Figure 4.A, during embryonic development the number of LCN2-positive cells markedly increased in the liver of control embryos (whose progenitor was injected with vehicle). We extended this qualitative observation by counting the number of LCN2 positive cells and confirmed that cells that present LCN2 positive signal increase with embryonic age (Figure 4.B). Some of the immunoreactive cells presented a nucleus with a multilobulated shape that is characteristic of neutrophils (Figure 5.A, 5.B). No hepatocyte appears to be stained. Of notice, LCN2 positive cell count in embryos whose progenitors were injected with LPS is slightly inferior to the control counterparts, although the difference is not statistically significant (Figure 4.B). Importantly, we failed to obtain viable embryos of embryonic age E11,5 and 19,5 from pregnant mice that were injected with LPS. It has been reported that pregnant females of some mice strains, C57BL/6 included, are susceptible to the effects of inflammatory agents such as LPS. The intraperitoneal administration of LPS in early stages of pregnancy can result in the resorption of the embryos, while the same stimulus in later gestational periods can lead to abortion (Xu et al. 2007; Role and Labor 2006; Robertson et al. 2007). This might explain the failure to obtain viable embryos from females that were injected with LPS.

After birth, animals that were injected with saline presented a significant number of LCN2-positive cells which persisted until P7 (Figure 6). From P16 onwards, LCN2 positive cells were rarely observed in the liver of control animals (Figures 6 and 7).

Identical to what was observed in embryonic samples, we detected neutrophil like cells in the liver of control newborn animals (Figure 5.A, 5.B). Furthermore, no hepatocytes appear to be stained. Structures typical of the developing organ such as erythroblastic islands were observed, but no LCN2 signal appears to be associated with them (Figure 5.A). Erythroblastic islands were no longer seen from P16 onwards.

The intra-peritoneal administration of LPS induced a ubiquitous LCN2 expression in the liver of all post-natal animals (Figure 6 and 7). The intensity of the signal through the different ages did not altered significantly, with all hepatocytes presenting LCN2 immunoreactivity. Besides hepatocytes, LCN2 immunoreactivity was also observed in neutrophil-like cells which were present in higher number when compared to control animals (Figure 4.B). We did not observed LCN2 staining in Kupffer-like cells in any of the LPS injected subjects (Figure 5.A).

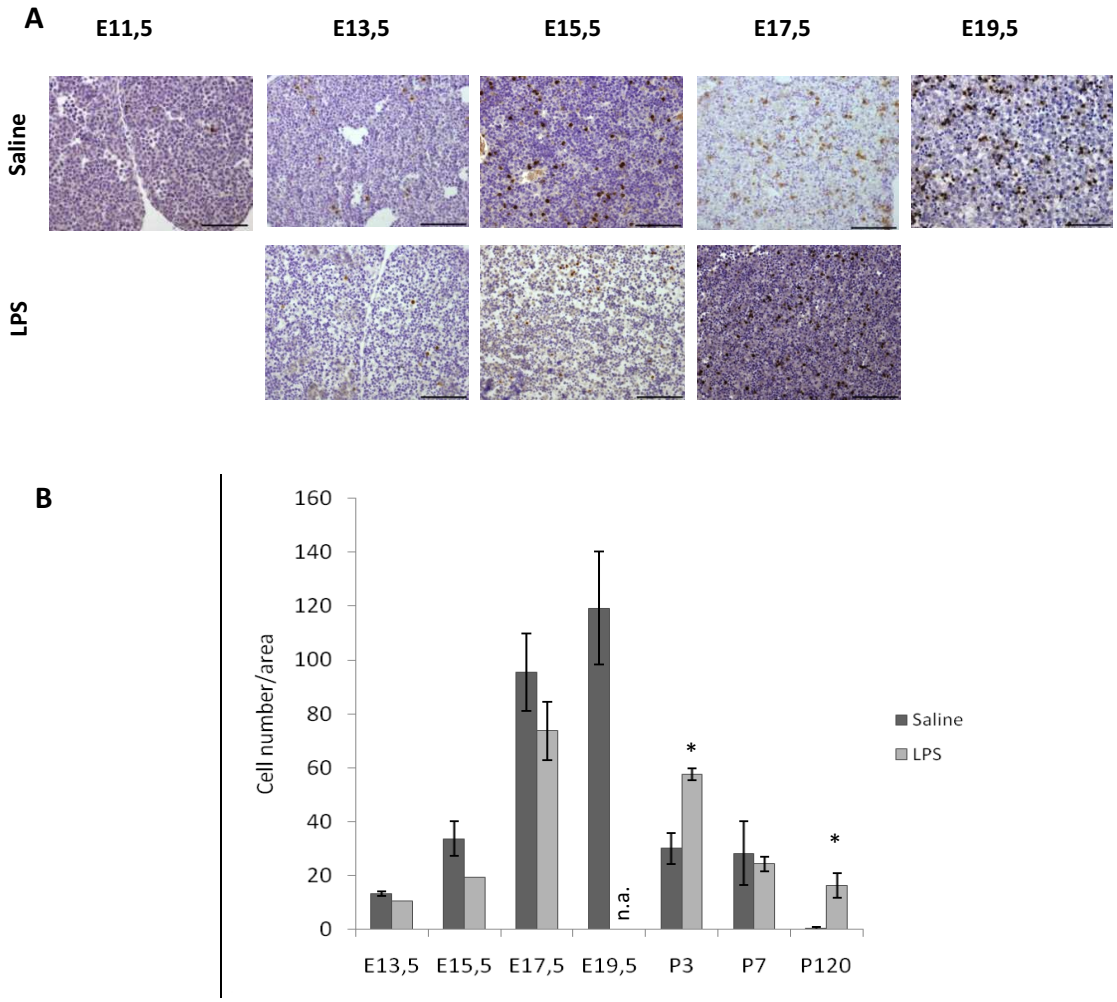

Figure 4 Immunohistochemistry for LCN2 in embryos and analysis of the number of LCN2 stained cells in the liver of liver of mice of embryonic and post natal time points. No viable embryos were obtained for the E11,5 and E19,5 time points of lipopolysaccharide (LPS) injected females. **(A) (Upper panel)** LCN2 staining cells in the liver of embryos whose progenitor was injected with saline increase in number throughout embryonic development. **(A) (Lower panel)** LPS administration in pregnant mice dams does not result in a evident change in the pattern of LCN2 stained cells, when compared with control animals. **(B)** Quantification of LCN2 stained cells in the liver reveals that the number of positive cells increases with age until E19,5. This increase is similar in embryos derived from LPS or saline injected pregnant females; nevertheless, LPS treated subjects display a lower number of LCN2 positive cells when compared with controls, although the difference is not statistical significant. After birth, the number of LCN2 expressing cells in saline injected animals decreases to residual numbers from P16 onwards. LPS administration results in a statistical significant increase, when compared to controls, in LCN2 positive cells in the liver, with the exception occurring in P7 animals. Given that LPS injection results in LCN2 signal in the hepatocytes, only non-hepatocyte cells that stained for LCN2 were counted. The trend in cell counts between control and LPS injected animals in P120 subjects is also noticed in the remaining post-natal time points. Series of 5 images per subject were taken in the liver parenchyma, covering an area of 0.149 mm2. For each condition the liver of 3 animals was analyzed, with the exception of the 13,5 and 15,5 embryos from LPS injected females (n=1). Scale bar = **(A)** 100 μm.

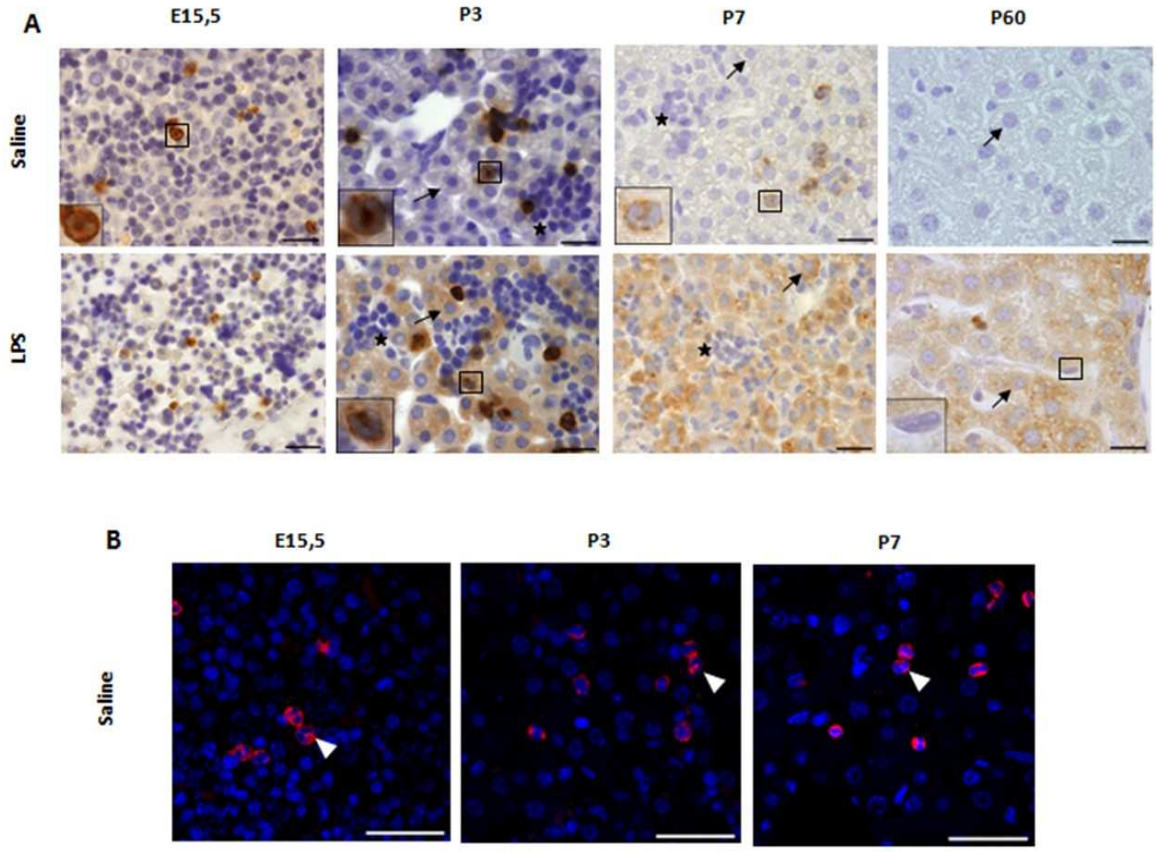

Figure 5 Identification of LCN2 expressing cells in the liver of control and LPS injected mice. (A) LCN2 immunoreactivity increases in the liver after LPS injection, when compared with the control counterparts. The exception occurs at embryonic stages. **(A) (Upper Panel)** LCN2 is not detected in hepatocytes (arrow) of saline injected animals in any of the studied age. Erythroblastic islands-like structures (star) are present in the liver until P7, and no LCN2 immunoreactivity in these cell clusters was observed. Some of the LCN2 positive cells have neutrophil like morphology, as can be seen in the insets. **(A) (Lower Panel)** The cell types that express LCN2 in the fetal livers of LPS injected pregnant dams is identical to what we observed in controls. On the other hand, LPS injection in post-natal animals results in overall LCN2 expression in the hepatocytes (arrow). As observed in saline treated animals, erythroblastic islands-like structures (star) are present and do not express LCN2. Neutrophil like cells displaying LCN2 immunoreactivity were also observed (P3, inset). Kupffer-like cells do not display LCN2 immunoreactivity (inset in P60 animal). (**B**) Immunofluorescence for LCN2 (Red) and the nuclear marker DAPI (Blue) confirm the presence of neutrophil like cells (arrowhead). Scale bar = (**A**) 20; (**B**) 50 µm.

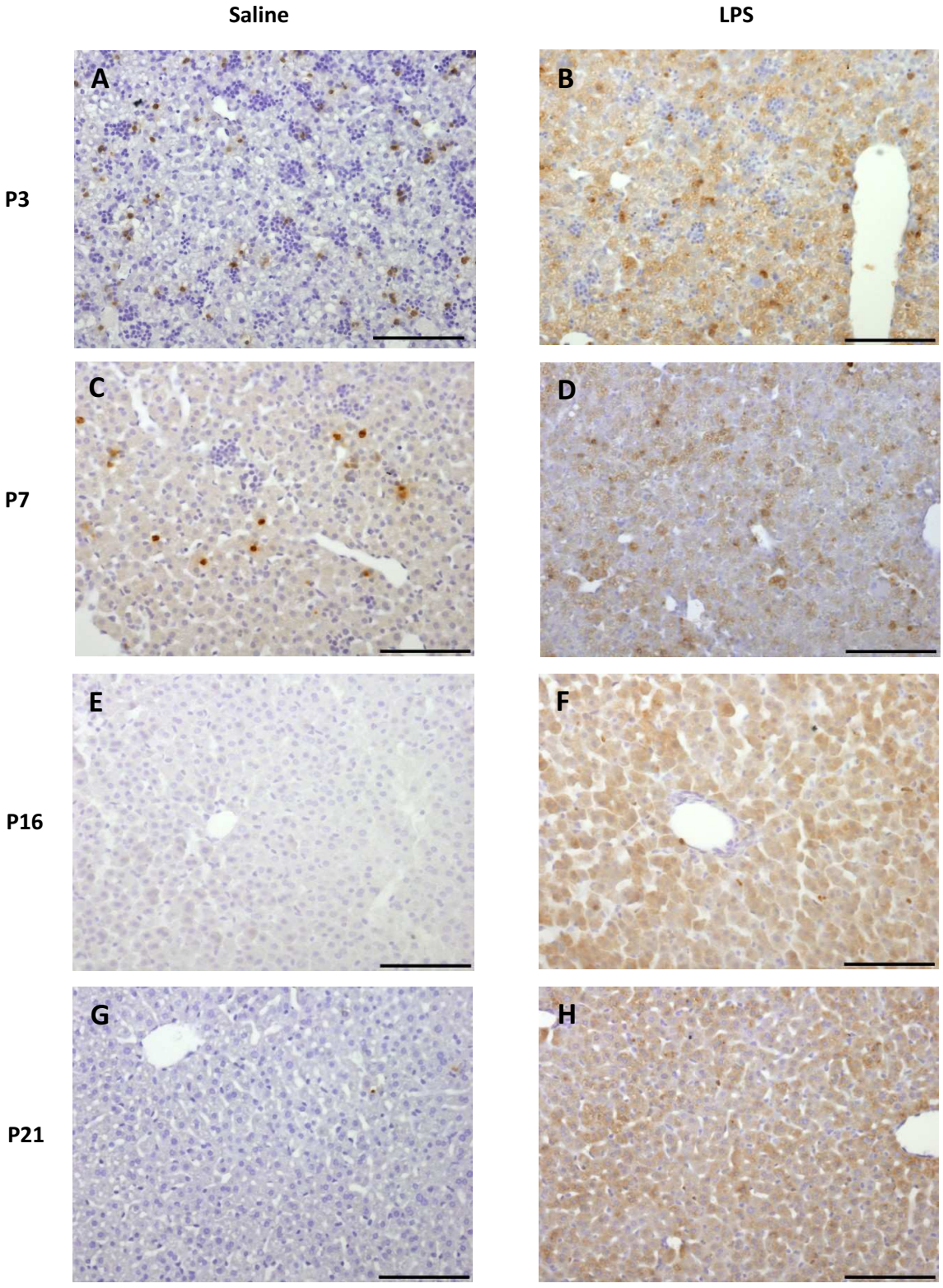

Figure 6 Immunodetection of LCN2 in the liver of post natal animals that were injected with saline or LPS. Only animals from pre-weaning ages P3 to P21 are shown. While LCN2 staining is evident in P3, it diminishes with age and from P16 onwards is rarely detected (**A**, **C**, **E**, **G**). On the other hand, LPS administration results in a broad LCN2 distribution in all analyzed ages (**B**, **D**, **F**, **H**). Scale bar = (**A** - **H**) 100 µm.

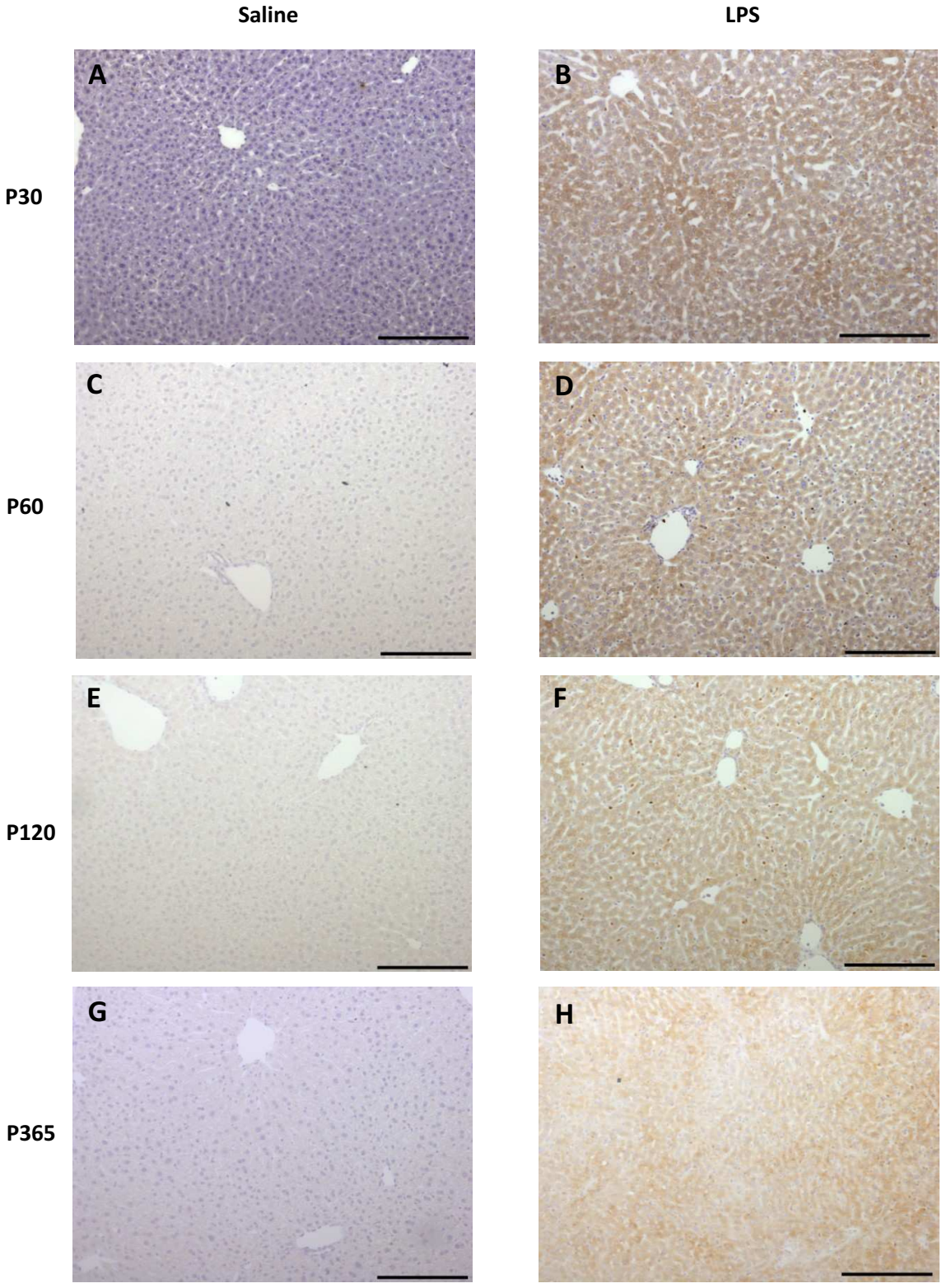

Figure 7 Immunodetection of LCN2 in the liver of adult animals that were injected with saline or LPS. Only animals from P30 to P365 time points are shown. No consistent LCN2 signal is detected in the liver of saline injected mice (**A**, **C**, **E**, **G**). As observed in younger animals, LPS injection leads to a ubiquitous expression of LCN2 in the liver of mice at least till one year of age (**B**, **D**, **F**, **H**). Scale bar = (**A** - **H**) 200 µm.

3.2. LCN2 expression pattern in the brain

During embryonic development, occasional staining for LCN2 was observed in the fetal brain but it was restricted to the stromal space of the CP in mice whose progenitor was injected with either saline or LPS (Figure 8.A).

In post natal animals injected with saline LCN2 expression in the brain was restricted to the CP stroma of 3 day old subjects. The older animals did not present any LCN2 signal in the brain (Figures 9 and 10). On the other hand, the peripheral inflammatory stimulus in post natal animals resulted in LCN2 immunoreactivity localized in the CP epithelia and stroma, blood vessels of the brain parenchyma and ependyma cell layer (Figure 8.B-E). This pattern was consistent in all of the post natal ages studied (Figures 9 and 10), with the notable exception of P3 animals, were no LCN2 signal was detected in CP epithelial cells (Figure 11.A). Immunofluorescence colocalization of LCN2 and transthyretin, a protein expressed specifically by the epithelial cells of the CP, further confirms that LCN2 is not expressed by the CP epithelial cells of 3 days old pups that were injected with LPS, while in older animals colocalization is evident in the CP epithelia (Figure 11.B-D).

Interestingly, the occurrence of the protein in LPS injected animals is heterogeneous through the epithelial cells layer of the CP, since not all cells present LCN2 signal. This pattern was seen in all of the post-natal time points from P7 onwards (Figure 9 and 10). We did not observed a clear age-dependent pattern in terms of LCN2 positive CP epithelial cells.

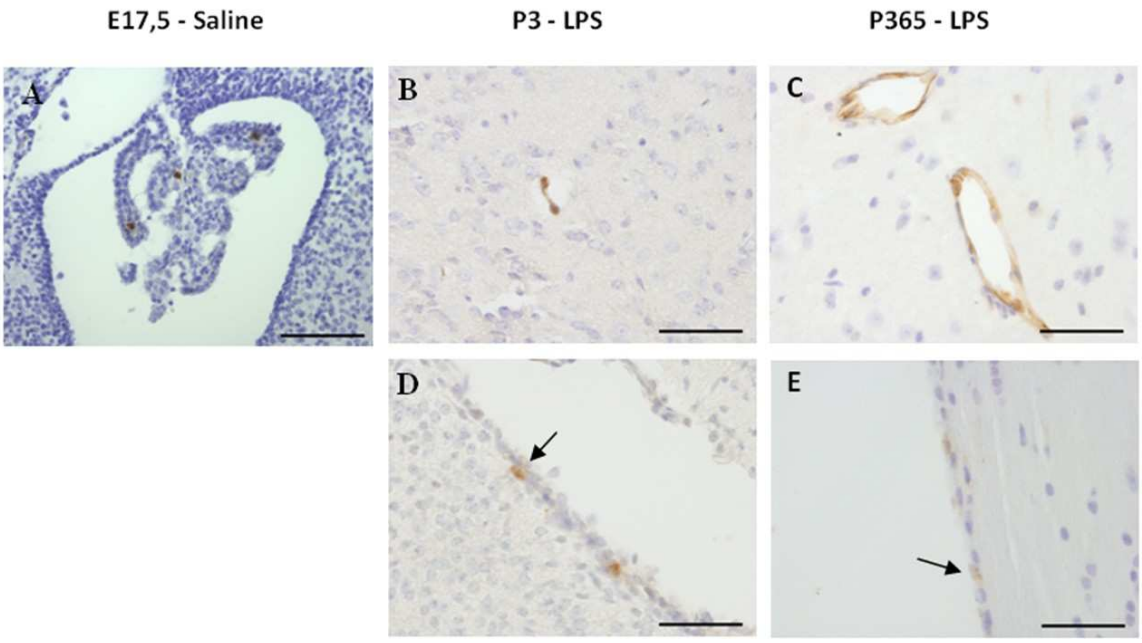

Figure 8 LCN2 immunoreactivity in the brain of control and LPS animals. LCN2 staining was restricted to the CP, ependymal layer lining the ventricles and blood vessels. (**A**) Occasional LCN2 staining was observed in the stromal space of some of the E17.5 embryos whose progenitor was injected with saline. Identical pattern occurred in some of the embryonic samples of other time points/injection. (**B**) In LPS injected mice endothelial cells of the blood vessels present in the brain parenchyma also stained for LCN2. (**C**) In older animals endothelial LCN2 signal was also observed. This pattern of expression was consistent through all of the studied post-natal time points. (**D**) LCN2 immunoreactivity was detected in ependymal cells (arrow) of 3 day old animals injected with LPS. (**E**) Similar to (**D**), LPS injection in year old animals results in occasional staining in cells of the ependyma layer (arrow). This pattern of LCN2 presence in the ependyma was observed in all post-natal time points. Scale bar = (**A)** 100; (**B**, **C)** 50 µm.

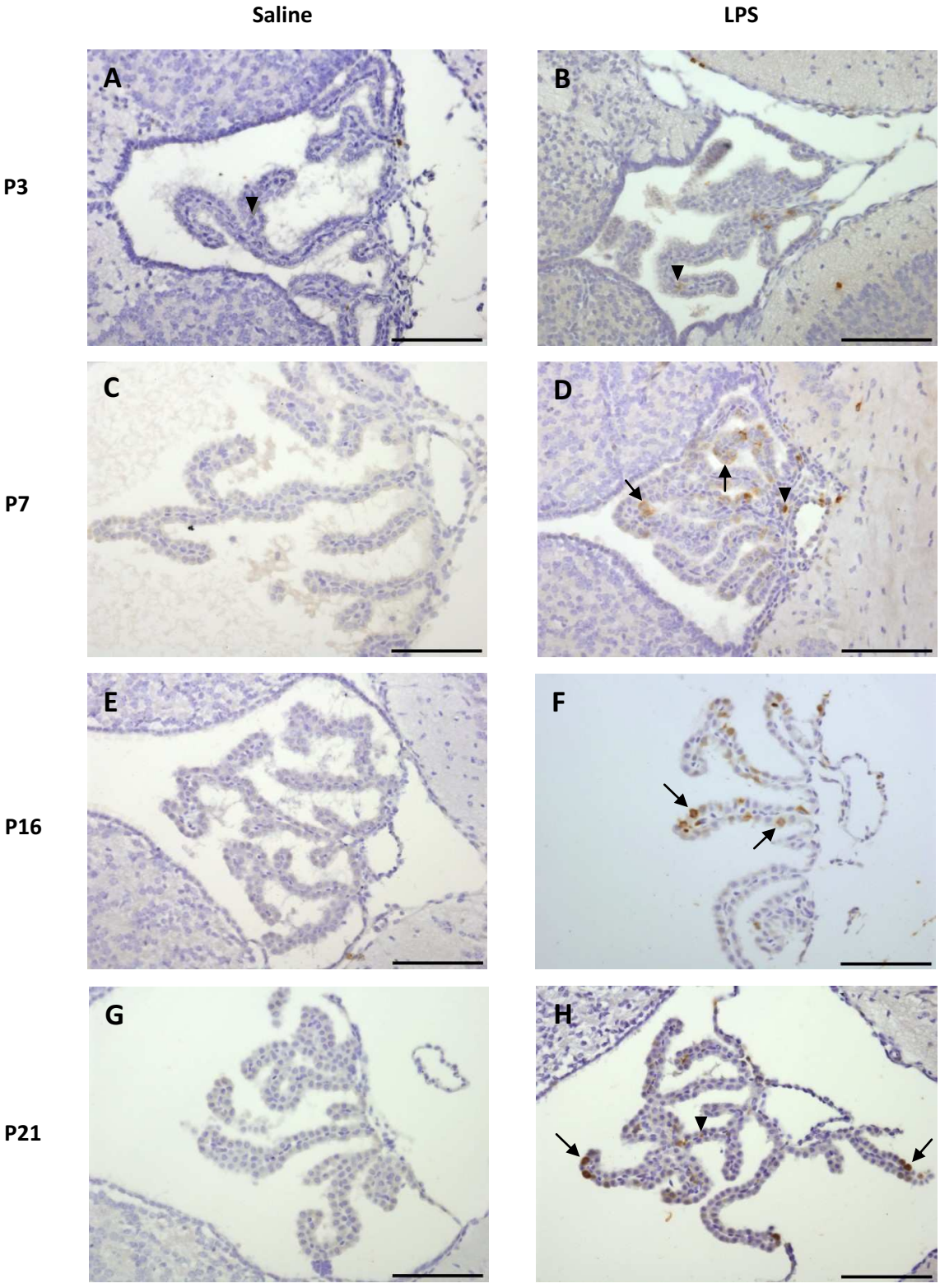

Figure 9 Immunohistochemistry for LCN2 in the choroid plexus of LPS and saline injected animals. Only animals from P3 to P21 are shown. The injection of LPS results in the presence of LCN2 staining in CP epithelial cells (arrow) from 7 day old animals onwards (**B**, **D**, **F**, **H**). Staining in the stromal area of the CP (arrowhead) occurs in all LPS injected animals. No LCN2 immunoreactivity is detected in saline controls (**A**, **C**, **E**, **G**). Scale bar = (**A**-**H**) 100 µm.

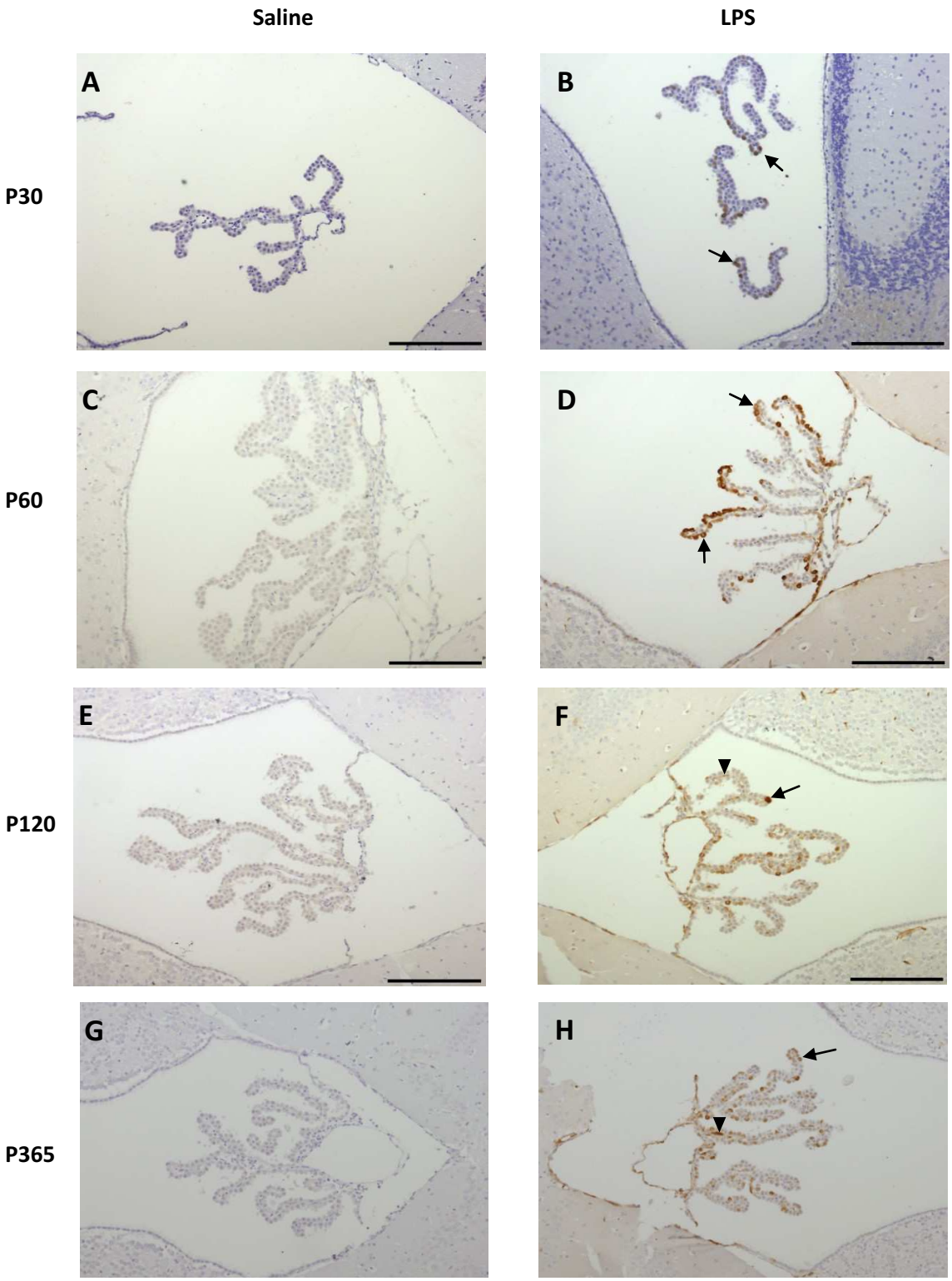

Figure 10 Immunodetection of LCN2 in the choroid plexus of LPS injected and saline animals. Only animals from P30 to P365 are shown. No staining for LCN2 is visible in control animals (**A**, **C**, **E**, **G**). LPS administration results in LCN2 detection in epithelial cells (arrow) and in stroma cells of the choroid plexus (arrowhead) (**B**, **D**, **F**, **H**). Scale bar = (**A**-**H**) 200 µm.

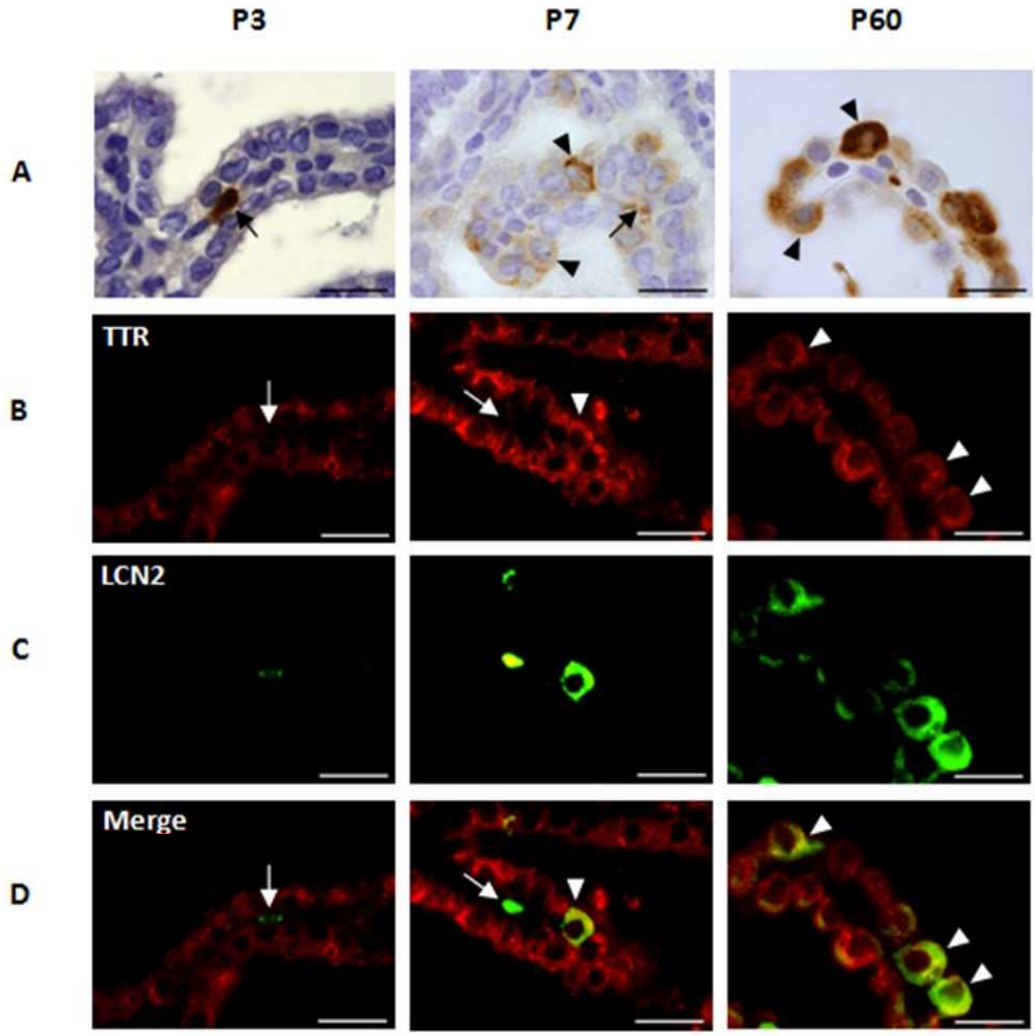

Figure 11 LCN2 localization in the Choroid Plexus of LPS injected animals. (**A**) Immunohistochemistry for LCN2 demonstrates that in P3 animals the protein signal is restricted to the stromal space (arrow). From P7 onwards, LCN2 is also present in the epithelial cells of the CP (arrowhead). (**B**, **C** and **D**) Immunofluorescence against LCN2 (**C**) and transthyretin (TTR), a protein produced only by CP epithelial cells (B), confirms that the expression of LCN2 in CP epithelial cells (arrowhead) of LPS injected animals only occurs from P7 onwards, as the merging of (**B**) and (**C**) reveals (**D**). Scale bar = (**A**-**D**) 20 μm.

3.3. LCN2 expression pattern in the spleen

The study of the expression pattern of LCN2 in the spleen was confined to the post-gestational period since this organ was not observed in all embryos analyzed. In the post-natal spleen the expression of LCN2 was detected in all analyzed ages in both saline (control) and LPS treated animals.

In the spleen of post-natal control animals the presence of LCN2 was mainly restricted to cells in the red pulp region. This expression pattern was generally consistent throughout age (Figures 12 and 13), although the intensity of LCN2 positive staining in P3 animals was slightly weaker than the one observed in older control animals. LCN2 positive cells in the white pulp area were rarely observed. It is important to notice that a clear histological distinction of the white pulp from the surrounding tissue was not possible in the P3 and P7 animals. Thus we did not characterize the LCN2 expression profile in the white pulp of those specimens.

The LPS injection resulted in a notable increase of LCN2 positive cell number in the red pulp area of the spleen, when compared with the same area of saline injected animals. This observation is consistent for all of the post-natal time points analyzed (Figure 12 and 13). The analysis of the number of LCN2 positive cells in the red pulp region of animals of different ages confirmed that this increase in LCN2 positive cells after the inflammatory stimulus occurs independently of the animal age since LCN2-positive cells numbers are identical through the different ages (Figure 14). Similarly, the cell numbers of control animals do not present significant differences between the different ages. The inflammatory stimulus also resulted in a higher occurrence of LCN2 stained cells in the white pulp area of the spleen when compared with controls, despite the low number of positive cells in this region of the spleen. This pattern was consistent in all studied time points (with the exception of P3 and P7 subjects for the reasons referred above), although we only depict the P60 time point in Figure 15.

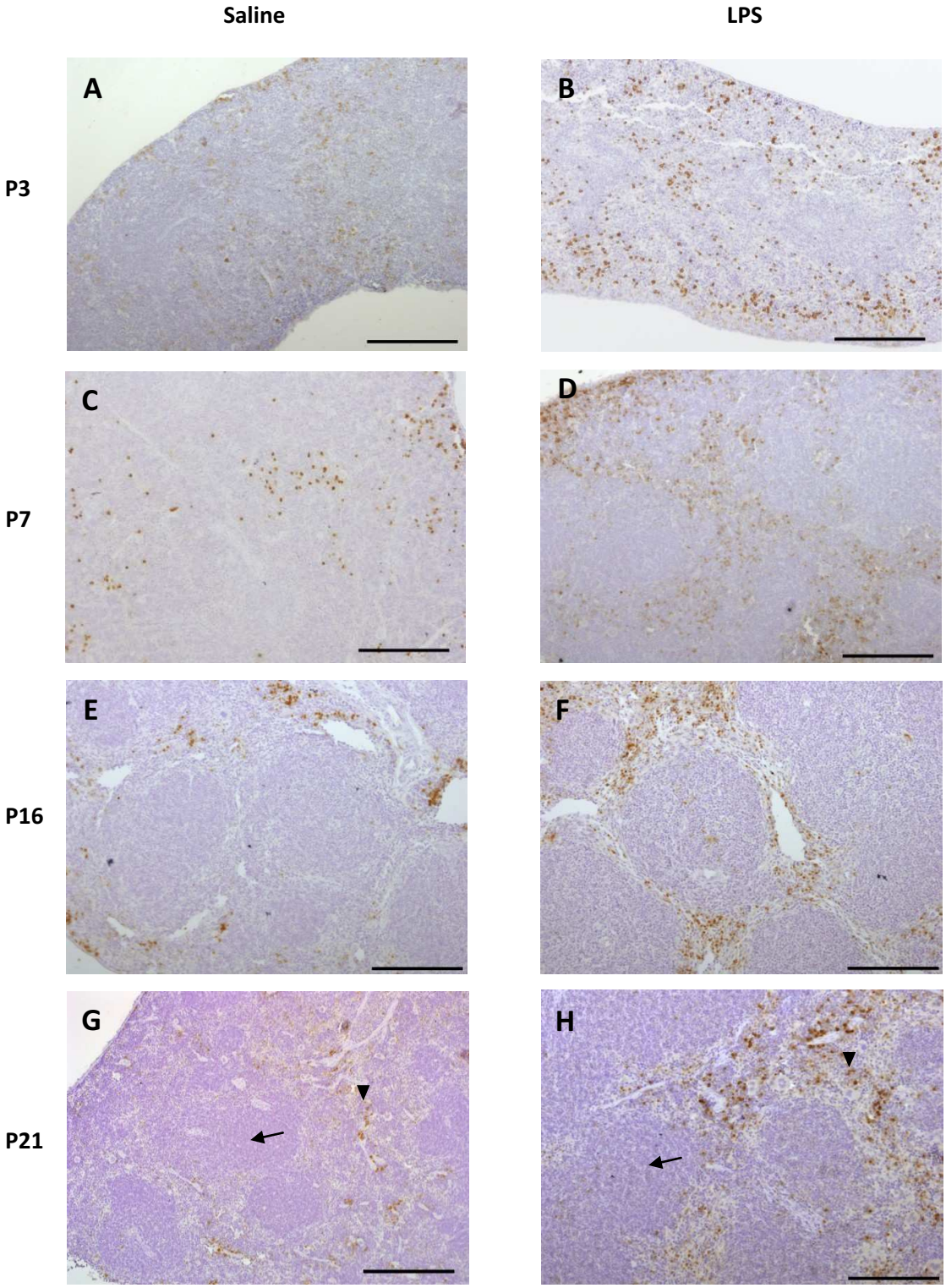

Figure 12 Immunodetection of LCN2 in the spleen of saline and LPS injected animals. Only animals from P3 to P21 are shown. In control animals LCN2 positive cells are present in the red pulp area (arrowhead), with the staining in the white pulp area being practically absent (**A**, **C**, **E**, **G**). The LPS inflammatory stimuli result in the increase of LCN2 positive cells in the red pulp area of the organ (arrowhead) (**B**, **D**, **F**, **H**). Occasional LCN2 positive cells are also present in higher number in the white pulp area of the spleen of LPS injected animals (arrow in H). Scale bar = (**A**-**H**) 200 μm.

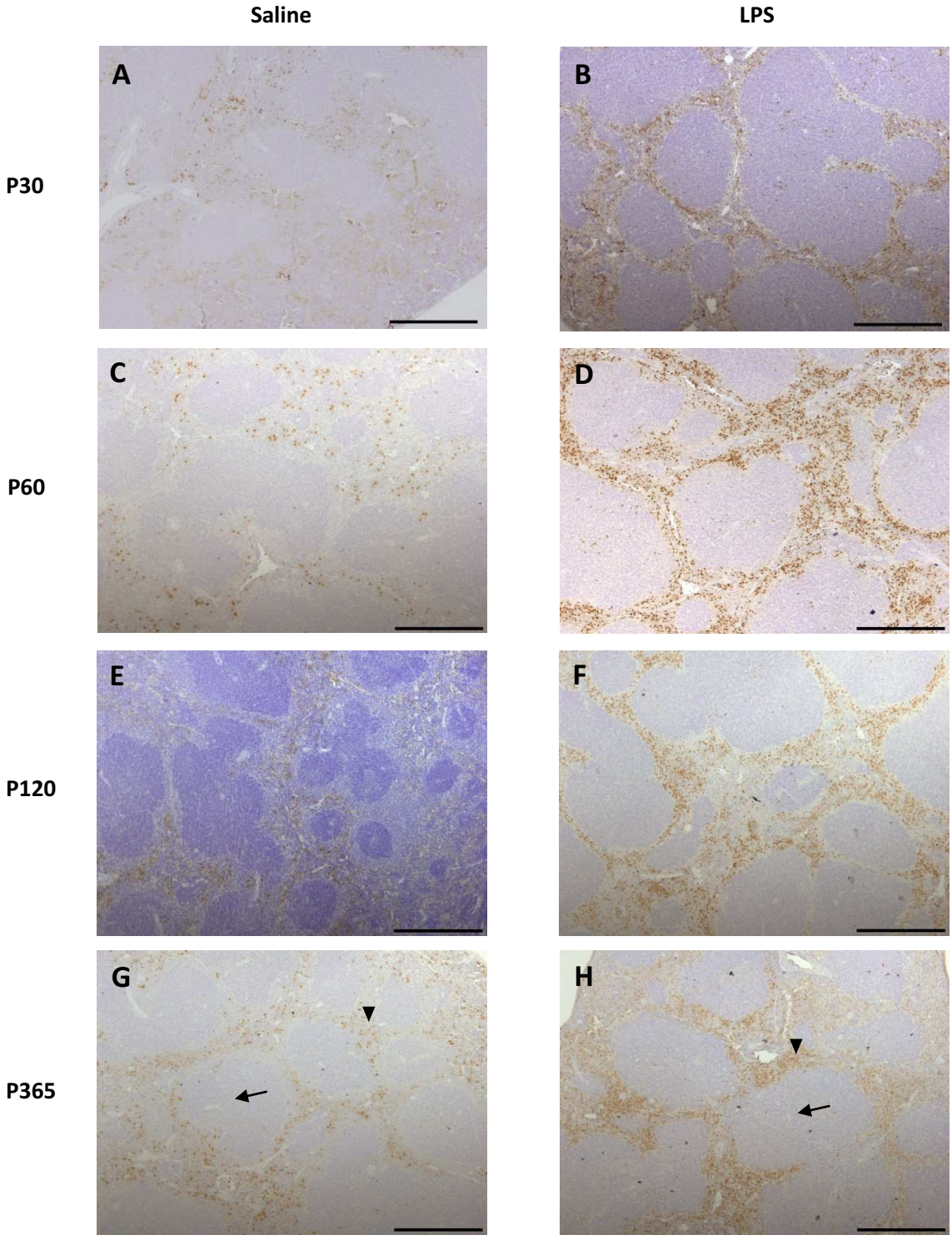

Figure 13 Immunohistochemistry for LCN2 in the spleen of saline and LPS injected animals. Only animals from P30 to P365 are shown. Identically to what was observed in younger animals, saline injection results in the detection of some LCN2 positive cells, namely in the red pulp area (arrowhead in **G**) of the organ, with staining in the white pulp (arrow in G) area being practically non-existent (**A**, **C**, **E**, **G**). LPS administration results in an overall increase in the number of LCN2 positive cells, both in the red pulp area (arrowhead in H) and in the white pulp area (arrow in H) of the organ (**B**, **D**, **F**, **H**). Scale bar = (**A**-**H**) 500 µm.

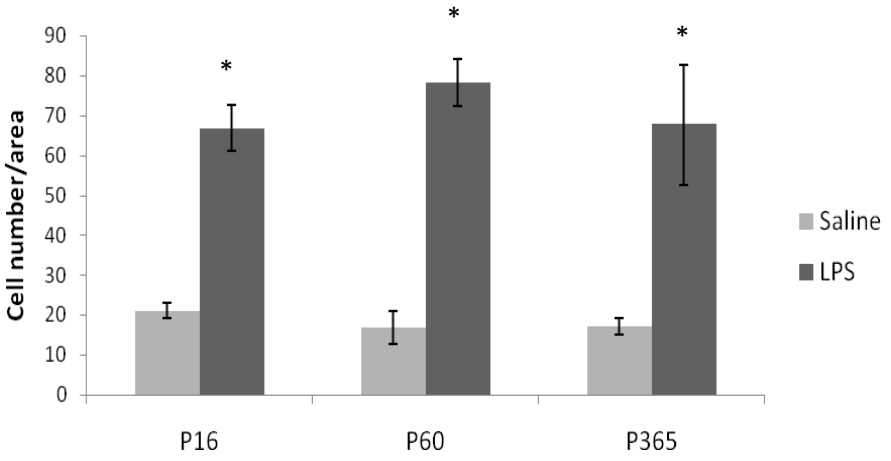

Figure 14 Number of LCN2 positive cells in the spleen of animals of different ages. In saline injected animals the cell number does not change significantly with age. LPS administration results in a significant increase in the number of LCN2 immunoreactive cells when compared with controls but there is no significant variation between animals of the LPS-injected group. Series of 5micrographs per subject were taken in the organ red pulp, with each photo covering an area of 0.0367 mm2. For each condition the spleen of 3 animals was analyzed.

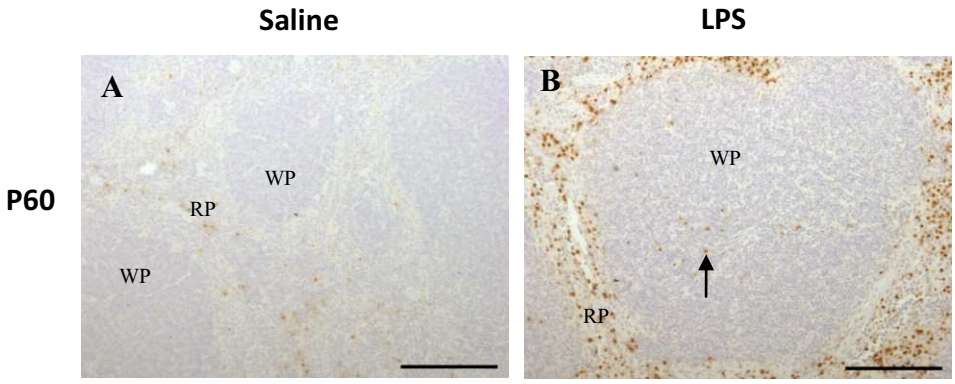

Figure 15 Localization of LCN2 immunoreactivity in the spleen of 60 days old animal injected with either LPS or saline. (**A**) In control animals LCN2 staining in the white pulp (WP) is practically non-existent, while in the red pulp region (RP) cells positive for the protein are found scattered. (**B**) LPS administration results in a notorious increase of LCN2 stained cells in the red pulp area (RP) of the organ. Some cells that stain for LCN2 (arrow) are present in the splenic white pulp (WP). This pattern of expression is consistent through all of the studied post-natal time points. Scale bar = (**A**,**B**) 200 μm.

3.4. LCN2 expression pattern in the kidney

LCN2 presence in the kidney was first detected in late embryonic development, more specifically from E17,5 onwards. The protein appears to be localized in tubular structures (Figure 16), whilst the glomeruli do not present LCN2 immunoreactivity. To identify with more precision the structures that present LCN2 positive signal, we counterstained the slides with periodic acid-Schiff (PAS). PAS is a histological technique that stains complex carbohydrates in magenta. In the kidney, PAS stains mainly the basement membrane which supports the tubular epithelium and the brush border that projects into the lumen of proximal tubules (PT). This staining technique is useful for a clearer distinction between proximal and distal tubules (DT), since DT do not possess brush border, thus they do not present the typical magenta staining in the apical side of the tubules. Counterstaining with PAS revealed that the LCN2 staining in the kidney of E17,5 embryos from saline injected females appears to occur mainly in PT (Figure 16.A3). Of notice, not all of the PT display LCN2 signal. This pattern of expression persists in post natal animals injected with saline (Figure 17 and 18), although from P16 onwards the LCN2 immunostaining in these structures is usually very faint (Figure 16.C3). Interestingly, whereas in the embryo and newborn kidney this LCN2 staining is distributed in the cytoplasm of PT cells, from P16 onwards the staining is localized at the apical site of the tubules (Figure 16.A2, 16.C3). Furthermore, little to no LCN2 immunoreactivity was detected in the nephrogenic zone (NZ) of the newborn animals, either P3 or P7. The NZ, where nephron differentiation occurs, is visible at the periphery of the organ and structures typical of the area such as ureteric buds are visible (Figure 16.B1).

LPS administration resulted in a clear and strong immunoreactivity in the kidney of all post-natal animals (Figure 17 and 18). In LPS injected newborn mice the LCN2 signal intensity and presence was higher when compared with the respective controls (saline injected newborns). Of interest, the tubular structures that display LCN2 expression are the same both in LPS and saline injected newborns.

LCN2 signal was present in the cortical area of all of the post-natal time points of LPS injected animals. The cells expressing LCN2 belong mainly to PT-like structures (Figure 16.C2, 16.D2). Furthermore, the expression appeared to occur with more intensity in the apical side of the PT (Figure 16.C1, 16.D1). Of notice, similarly to what was described for control animals, not all of the PT presented LCN2 staining. Immunoreactivity

in PT that is continuous with the renal corpuscle was sporadically observed (Figure 16.D1). Interestingly, from P60 onwards LCN2 staining also appeared in the medullar portion of the organ, mainly in collecting ducts-like structures. Furthermore, the incidence of LCN2 stained structures in the medulla increased with age (Figure 18). Hence, while in the two month old animals the LCN2 signal is scattered through the medulla, in P120 subjects a significant proportion of the filtrate collecting structures are LCN2 positive and in P365 animals most of the medullar area displays LCN2 immunoreactivity (Figure 16.D3). Simultaneously, the frequency of cortical DT that exhibits LCN2 staining increases from P60 onwards, with the majority of the DT of P365 animals presenting LCN2 immunoreactivity (Figure 16.D2).

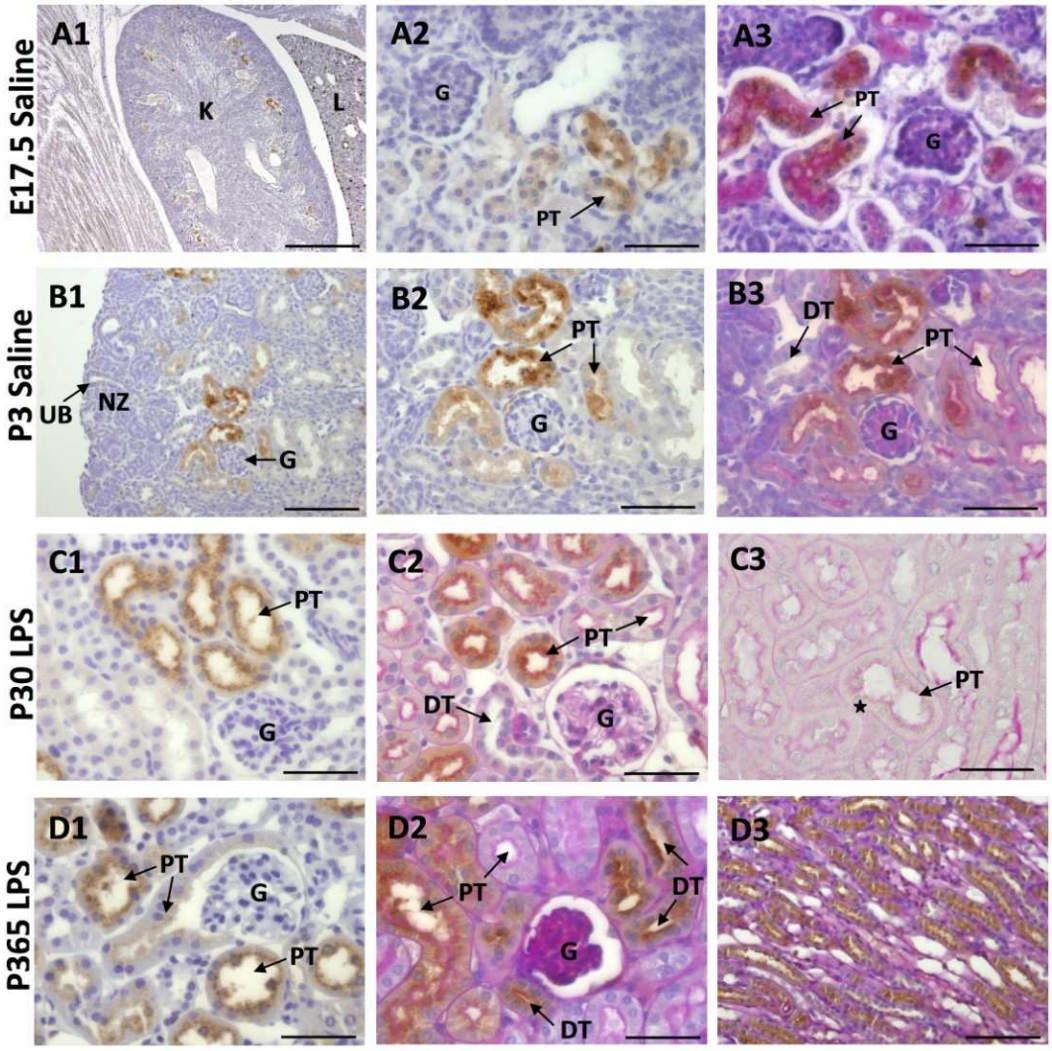

Figure 16 Morphological analysis of kidney LCN2 expression. The animals/images presented are representative of the main LCN2 staining trends observed in the kidney. (**A1**) LCN2 signal was found scattered in the kidney of E17,5 mice whose progenitor was injected with saline. (**A2**) Higher magnification of (**A1**) shows specific LCN2 staining in proximal tubules (PT)-like structures. No staining is detected in the glomeruli (G). (**A3**) Counterstaining with PAS confirms that LCN2 staining occurs in the PT, which presents magenta toned apical brush borders. (**B1**) LCN2 signal is restricted to the cortical area of the kidney in 3 day old pups injected with saline. No LCN2 stain is observed in the nephrogenic zone (NZ) of the organ, where structures that will develop into the mature nephron are recognizable, such as the ureteric bud (UB). (**B2**) Magnification of A1 suggests that the LCN2 stained cells are part of the PT. (**B3**) (**C1**) LPS injection results in the LCN2 immunostaining in PT like structures. (**C2**) Counterstaining with PAS confirms the +previous observations. Of notice, LCN2 presence is concentrated in the apical side of the PT. No LCN2 immunoreactivity is observed in distal tubules (DT). (**C3**) Cortical magnification of a saline injected kidney, counterstained with PAS. Faint LCN2 signal is observed in some PT structures (star). (**D1**) Similarly to C1, LPS administration resulted in an apical LCN2 signal in PT-like structures. A weak LCN2 immunoreactivity is visible in the PT that is continuous with the glomeruli (G). (**D2**) Counterstaining with PAS confirmed the previous observations. Of notice, distal tubules (DT) also display LCN2 signal. (**D3**) Medullar portion of the organ counterstained with PAS. The majority of collecting ducts-like structures present LCN2 immunostaining. Scale bar = (**A1**) 500; (**B1**, **D1**) 100; (**A2, A3, B2, B3, C1-D2**) 50; (**D3**) 100 μm.

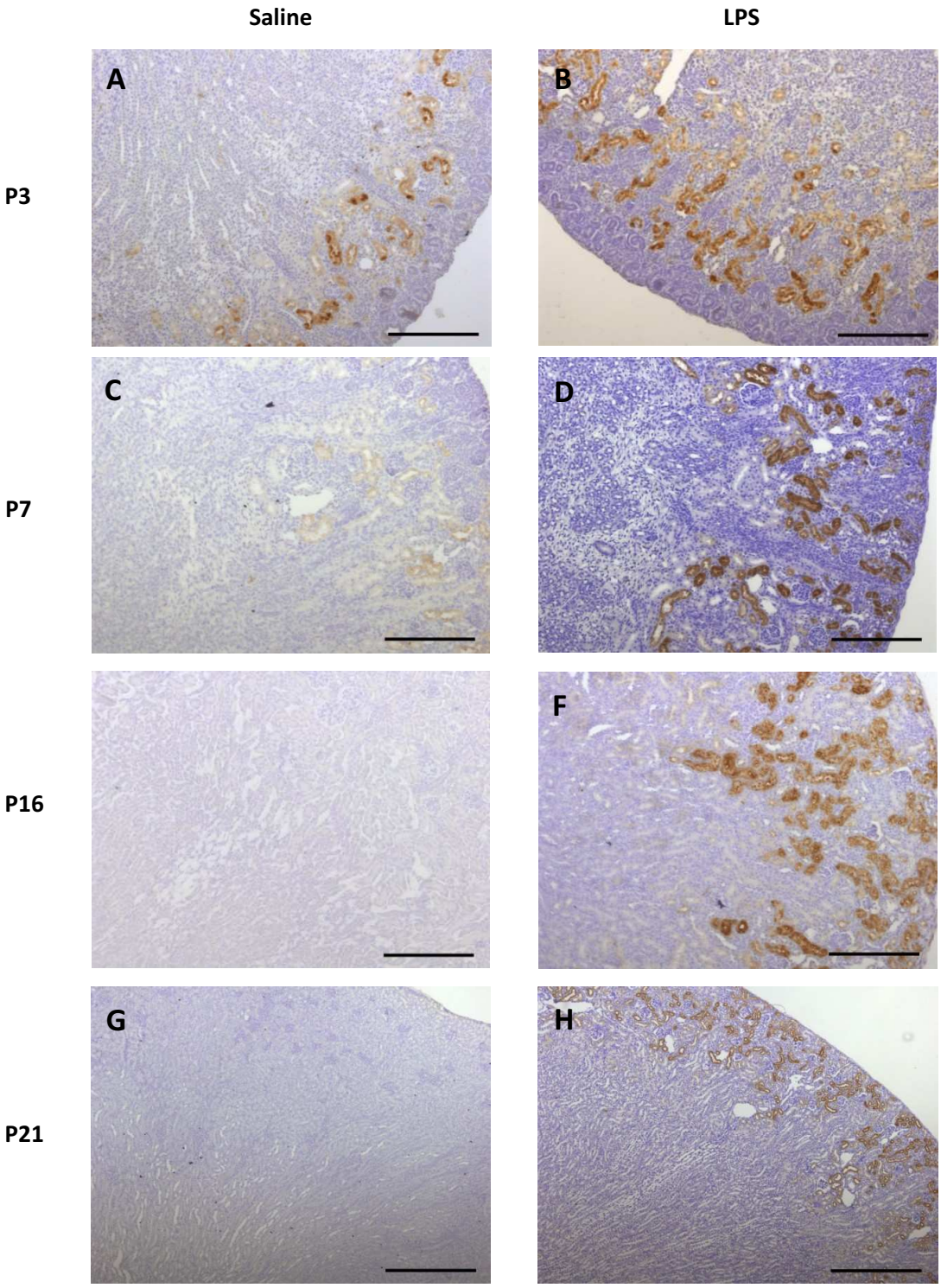

Figure 17 Immunohistochemistry for LCN2 in the kidney of LPS and saline injected mice. Only animals from P3 to P21 are shown. In the control animals LCN2 presence was mainly detected in the cortex of P3 and P7 subjects (**A**, **C**). From P16 onwards faint LCN2 signal is detected in the cortex of saline injected subjects (**E, G**). LPS administration results in an overall increase of LCN2 positive cells in the cortical area of the organ (**B, D, F, H**). Scale bar = (**A** – **F**) 200; (**G**, **H**) 500 μm.

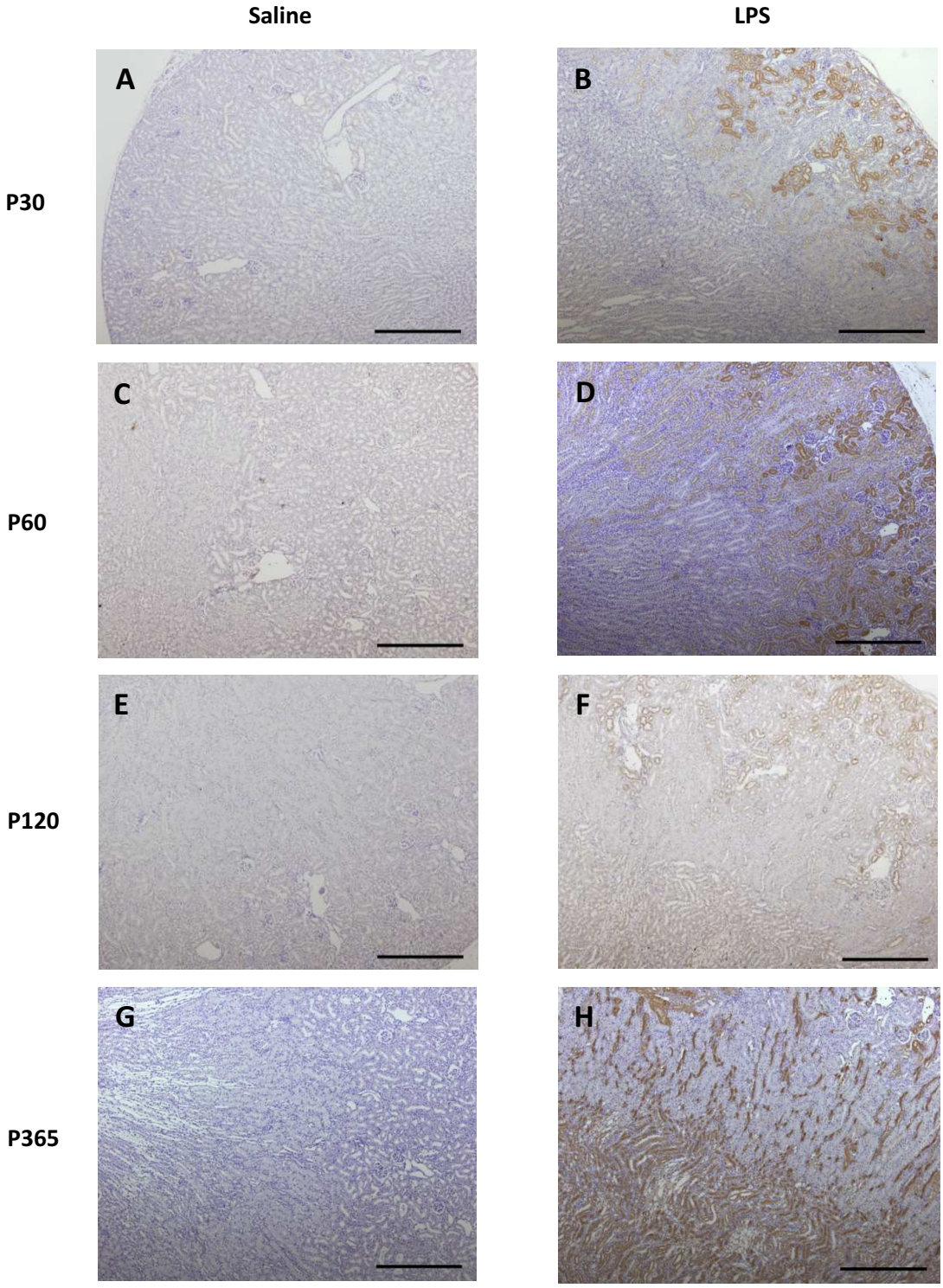

Figure 18 Immunodetection of LCN2 in the kidney of LPS and saline injected mice. Only animals from P30 to P365 are shown. In saline injected animals faint LCN2 expression is detected in the cortical area of the kidney (**A, C**, **E, G**). LPS injection leads to a significant LCN2 immunoreactivity (**B, D, F, H**), namely in the cortical area of the organ. In older animals, the majority of cells of the medullar portion are LCN2 positive (**F, H**). Scale bar = (**A**-**H**) 500 μm.

3.5. LCN2 expression pattern in the thymus

Of all of the organs analyzed for the presence of LCN2, the thymus appeared to be the most unaffected by the inflammatory stimulus. LPS administration did not appear to alter the pattern of expression of LCN2 in the thymus when compared with the saline counterparts (Figure 19 and 20). The presence of LCN2 stained cells was observed from the first post natal time point (P3) onwards, in both LPS and saline injected mice. In P3 animals the number of LCN2 positive cells was scarce. The number of LCN2 positive cells increased slightly in P7 animals to then appear more frequently in the animals with ages from P16 to P60. In older animals, P120 and P365, the distribution of the LCN2 positive cells was reduced. Interestingly, in all analyzed time points the LCN2 positive cells were present both in the medulla and cortex of the organ.

It is known that the thymus atrophies with age, with the thymic tissue being replaced by fat (Savino 2000; Sutherland et al. 2005). We had difficulties in obtaining structurally well conserved thymus from the one year old animals, and while we were able to find a consistent pattern of LCN2 expression in the organ of saline injected P365 animals, the same was not true for the LPS counterparts. Different patterns of LCN2 expression were observed in the thymus of these P365 LPS injected animals, ranging from residual to frequent LCN2 staining of the thymic cells. Hence, the analysis of LCN2 expression in this particular time point and injection is not conclusive. Nevertheless, in Figure 20.H a micrograph representative of the observed organs can be observed, with scattered LCN2 staining in the medulla and cortex of the organ.

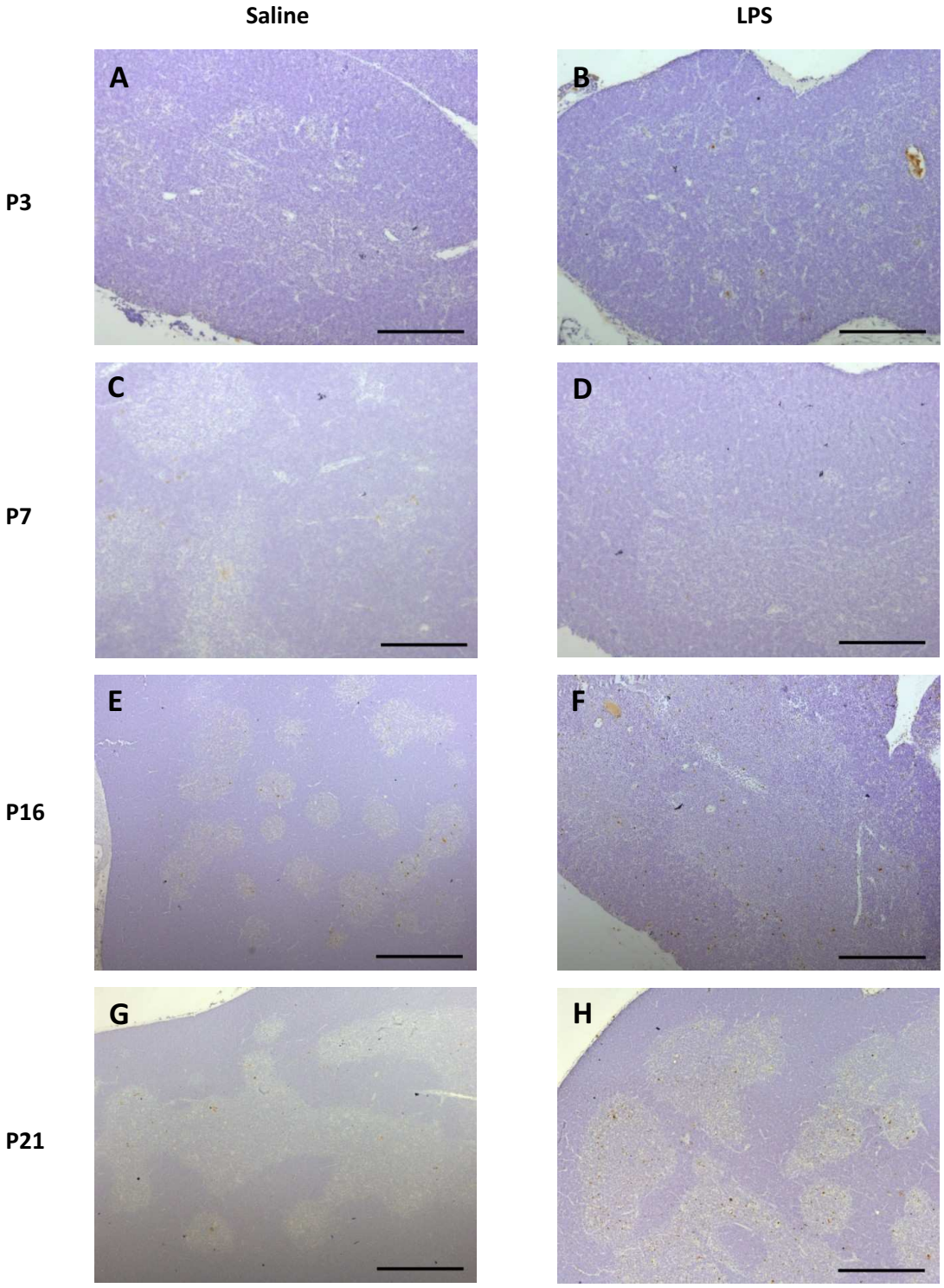

Figure 19 Immunodetection of LCN2 in thymus of saline and LPS injected mice. Only animals from P3 to P21 are shown. No difference in the distribution of LCN2 positive cells between LPS and control counterparts is observed. The LCN2 stained cells are scarce in P3 (**A**, **B**), their number increase in P7 (**C**, **D**) and are found scattered in P16 and P21 (**E - H**). The distribution of the immunoreactive cells appears identical between the cortex and the medulla of the organ. Scale bar = (**A - D**) 200; (**E – H**) 500 µm.

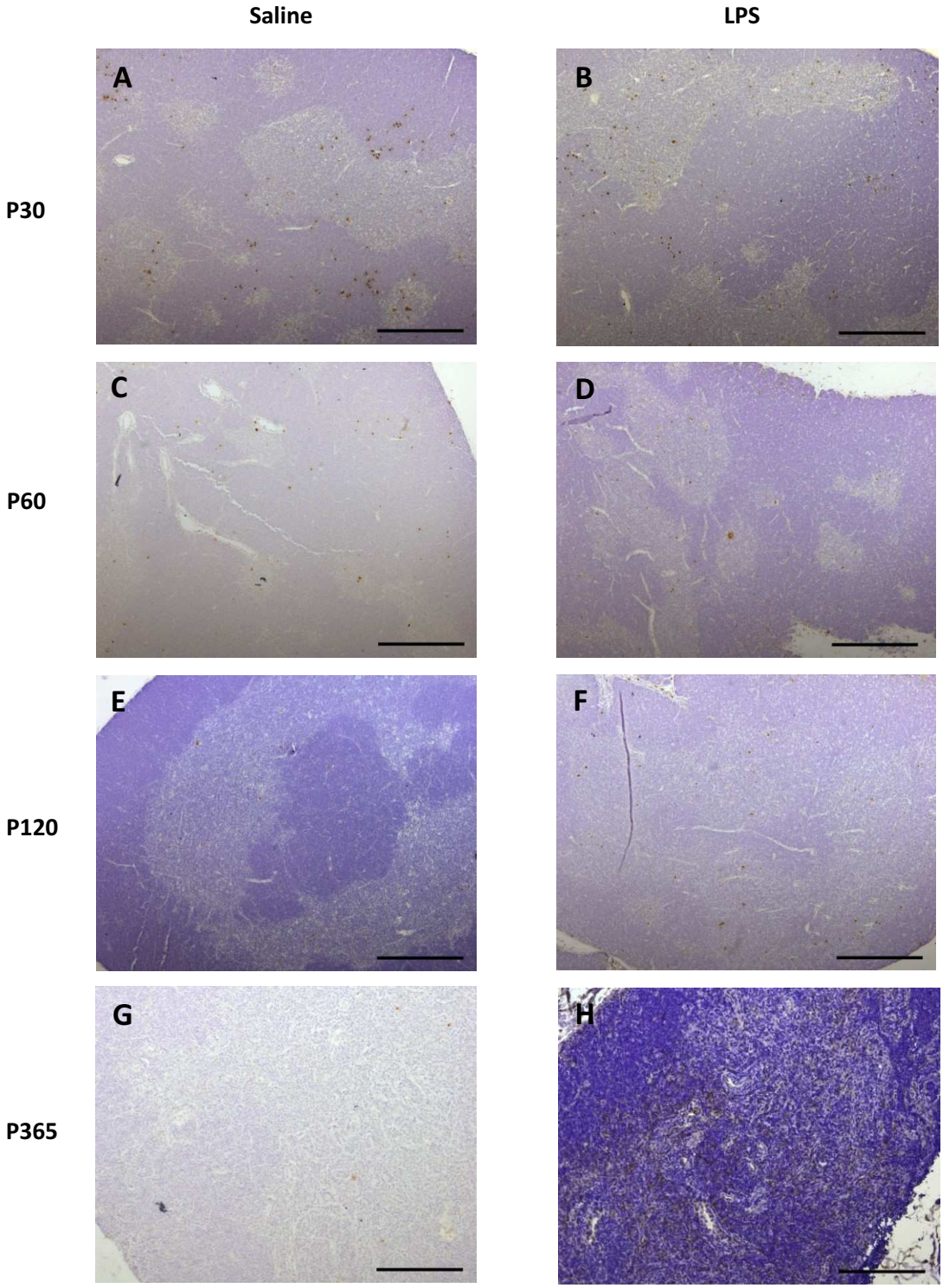

Figure 20 Immunohistochemistry for LCN2 in the thymus of saline and LPS injected mice. Only animals from P30 to P365 are shown. No evident difference in the distribution of LCN2 positive cells between LPS and control counterparts. Some LCN2 positive cells are found scattered in P30 and P60 subjects (**A** - **D**), but identically distributed between the cortex and the medulla of the organ. A reduction in LCN2 stained cells is noticed from P120 onwards (**E** – **G**). (**H**) Although there was not a clear pattern of LCN2 presence in the P365 animals injected with LPS. Scale bar = (**A** - **F**) 500; (**G**, **H**) 200 μm.

3.6. LCN2 expression profile summary

Bellow we present an overview of the LCN2 expression pattern in the studied tissues of all post-natal animals.

Table 1 Expression pattern of LCN2 in the studied organs of post-natal animals.

Relative number of LCN2 stained cells (# cells): (-), none; (+), sporadic; (++), scattered; (+++), frequent; (++++), all or practically all cells.

Intensity of LCN2 staining (stain): (-), none; (+), faint brown; (++), medium stain; (+++), strong stain; (++++), heavy staining

* A clear histological distinction of the white pulp was not possible in these spleens.

No clear pattern of LCN2 signal was identified in the thymus of P365 animals injected with LPS

		Kidney cortex		Kidney medulla		Thymus cortex		Thymus medulla		Liver		Brain (CP)		Spleen red pulp		Spleen white pulp	
Injection	Animal	#cells	stain	#cells	stain	#cells	stain	#cells	stain	#cells	stain	#cells	stain	#cells	stain	#cells	stain
LPS	P3	+++	+++	-	-	-	-	-	-	+++	+++	-	-	+++	++++	*	*
	P7	+++	+++	-	-	+	++	+	++	+++	+++	++	+++	+++	++++	*	*
	P15	+++	+++	-	-	++	++++	++	+++	++++	+++	++	+++	+++	++++	+	+++
	P21	+++	+++	-	-	++	+++	++	+++	++++	+++	++	+++	+++	++++	+	+++
	P30	+++	+++	-	-	++	+++	++	+++	++++	+++	++	+++	+++	++++	+	+++
	P60	+++	+++	++	++	++	+++	++	++	++++	+++	++	+++	+++	++++	+	+++
	P120	+++	+++	+++	++	+	+++	+	++	++++	+++	++	+++	+++	++++	+	+++
	P365	+++	+++	+++	++++	?	?	?	?	++++	+++	++	+++	+++	++++	+	+++
Saline	P3	++	+++	-	-	-	-	-	-	++	+++	-	-	++	+++	*	*
	P7	++	++	-	-	+	++	+	++	++	+++	-	-	++	+++	*	*
	P15	+	+	-	-	++	+++	++	+++	-	-	-	-	++	+++	-	-
	P21	+	+	-	-	++	+++	++	+++	-	-	-	-	++	+++	-	-
	P30	+	+	-	-	++	+++	++	+++	-	-	-	-	++	+++	-	-
	P60	+	+	-	-	++	+++	++	+++	-	-	-	-	++	+++	-	-
	P120	+	+	-	-	+	+++	+	+++	-	-	-	-	++	+++	-	-
	P365	+	+	-	-	+	++	+	++	-	-	-	-	++	+++	-	-

4

Discussion

In this study we characterize the ontogenic expression pattern of LCN2, a 25 kDa lipocalin protein, under physiological and acute inflammatory conditions (24 hours after a single bacterial lipopolysaccharide (LPS) injection). LPS is a constituent of the cell wall of Gram-negative bacteria that is capable to elicit acute inflammatory responses. Multiple molecules are modulated during the inflammatory response and there is a growing body of evidence that supports the participation of LCN2 in that response (Alpízar-Alpízar et al. 2009; Berger et al. 2006; Flo et al. 2004; Marques et al. 2008; Sunil et al. 2007).

We analyzed by immunohistochemistry the expression pattern of LCN2 at several embryonic time points (E11,5; E13,5; E15,5; E17,5; E19,5) and at various post-natal time points until adulthood (namely in the brain, liver, spleen, kidney, and thymus), both in physiological and inflammatory conditions. There are various reports that identify LCN2 expression in diverse tissues, under various conditions, in varied animals species and strains. However, it does not exist, to the best of our knowledge, a consistent description of the expression profile of the protein trough the mammal life span, under standardized conditions, either in physiological or inflammatory scenarios.

Hence, with this work we aim to contribute to a deeper knowledge of LCN2 presence and distribution through the mice lifetime, both in basal and in acute inflammatory conditions. Furthermore, with the construction of this expression profile we expect to contribute to a better understanding of the biological relevance of LCN2, namely in iron distribution and innate immunity.

Our work demonstrates that LCN2 expression is present but varies through mice lifetime, from early embryonic stages to adulthood. Furthermore, we show that LCN2 expression is highly dependent of the physiological state. We will next discuss in further detail the LCN2 expression pattern we described with this study. Since the LCN2 signal in the embryonic phase was concentrated in the fetal liver and kidney, we will address the pattern of expression of both fetal organs in the discussion of the corresponding tissue.

4.1. LCN2 in the developing liver

The liver was one of the embryonic organs where we detected a significant LCN2 presence. LCN2 was first detected in the liver of control E11,5 animals and from this time point the number of liver LCN2 positive cells increased significantly trough embryonic

development. After birth, the number of LCN2 positive cells significantly decreased in control mice and from P16 onwards, the presence of LCN2 positive cells in the liver was practically nonexistent. A subset of the observed LCN2 expressing cells displayed neutrophil like morphology. The presence of granulocytes in the fetal liver is not surprising, as the organ is the main site of blood cells formation during fetal development.

The production of blood cellular components, a process commonly designated by hematopoiesis, is a relatively well described phenomena and in the developing mouse is first observed in the extra-embryonic yolk sac around embryonic day 7,5 (E7,5). Hematopoiesis then occurs in a sequence of intraembryonic sites that include the aorta-gonad-mesonephros region, thymus, placenta, fetal liver, spleen and bone marrow (for review see Cumano and Godin (2007)). The fetal liver is seeded at around E11,5 with hematopoietic stem cells (HSC), the precursors of all blood cell lineages (Mikkola and Orkin 2006). The organ then becomes the major site of hematopoiesis until birth, when the bone marrow replaces it as the main site of the hematopoietic process (Orkin and Zon 2008).

The micro environment of the fetal liver favors the rapid expansion of the HSC and hematopoietic progenitor cells, along with the differentiation of erythrocytes, myeloid cells, and lymphocytes (Cumano and Godin 2007; Orelio et al. 2009; Tada et al. 2006). The hematopoietic activity in the mouse fetal liver reaches its peak between E14,5 and E16,5 to then decrease until it ceases shortly after birth. Accordingly, the cellular density of some of the blood cells differentiated in this organ decreases gradually until early days after birth (Ema and Nakauchi 2000; Huang et al. 2007; Sasaki and Sonoda 2000; Tada et al. 2006; Wolber et al. 2002). In this sense, the pattern of LCN2 cellular expression that we observed in the liver of control perinatal animals is roughly in accordance with the cellular expansion derived from the hematopoietic process in this organ. To the best of our knowledge there is no concrete data evidencing LCN2 presence in hematopoietic cells of the fetal liver. There are, however, some reports that describe LCN2 expression in different lineages of hematopoietic cells in other hematopoietic sites. For instance, Miharada et al.(2005) demonstrate the presence of LCN2 mRNA and protein in granuloid and erythroid progenitor cells isolated from the bone marrow of adult mice. Although at lower levels, they also detected LCN2 mRNA in immature hematopoietic progenitors (Miharada et al. 2005). These authors later reported LCN2 mRNA expression in monocyte/macrophage

lineage cells, granulocytic cells, erythroid cells and HSC isolated from human umbilical cord blood. They also demonstrated LCN2 expression at the protein level in granulocytic cells (Miharada et al. 2008). Taking this data in consideration it is plausible that, in addition to neutrophils, some of the herein reported cells expressing LCN2 in the liver of embryos and newborn mice might belong to different hematopoietic lineages that are found to develop in the organ. Interestingly, Miharada et al. (2005) also reported that incubation of mouse hematopoietic cells with LCN2 resulted in reduced monocyte/macrophage lineage proliferation. LCN2 also inhibited erythroid progenitor's differentiation and survival (Miharada et al. 2005). Identical influence of LCN2 in human erythroid and monocyte/macrophage lineages was reported later by Miharada et al. (2008). Other authors also describe LCN2 as a facilitator of hematopoietic cells apoptosis (Devireddy et al. 2001; Lin et al. 2005). These results suggest a possible role for LCN2 in the modulation of the hematopoietic process. Indeed, Miharada et al. (2008) theorizes that in physiological conditions, the serum levels of LCN2 might be sufficient to inhibit to some extent the survival/ proliferation of hematopoietic lineages such as erythroid and macrophages. However, in scenarios where additional hematopoiesis is required, it is possible that those progenitor cells become less susceptible to the LCN2 pro apoptotic effects, eventually due to increased expression of intracellular survival factors (Kehrer 2009; Miharada et al. 2008).

In apparent contrast with these observations, LCN2 deficient mice do not present a perceptible imbalance of blood cellular components (Berger et al. 2006; Flo et al. 2004), with some authors suggesting that in the absence of LCN2, alternative proteins may ensure the maintenance of adequate hematopoietic cell numbers (Miharada et al. 2005). Whether this putative LCN2 mediated control of hematopoiesis occurs in the fetal liver during embryonic development is unknown.

Our data suggest that the observed LCN2 expression in the liver of control embryo and newborn animals may be linked with the hematopoietic process. Despite that, it is most likely that not all of the LCN2 positive cells are of the hematopoietic type. Indeed, we report here that the numbers of LCN2 positive cells in the liver during the perinatal period increase until E19,5. Considering that the peak of the hematopoietic activity in the liver of mice is described as to occur between 14,5 and 16,5 days of pregnancy (Ema and Nakauchi 2000; Tada et al. 2006), it is unlikely that the herein reported increase in numbers of LCN2

positive cells in the final stages of pregnancy are exclusively derived of the hematopoietic process.

The above discussion regarding the LCN2 expression in the fetal liver was focused in the results obtained from embryos of saline injected females. We had difficulties in obtaining viable dams from LPS injected females, as many of the embryos were either aborted or resorpted. Indeed, we were not able to retrieve a single embryo of E11,5 and E19,5 from LPS injected females. Negative effects of LPS in developmental outcome has already been described (Role and Labor 2006; Xu et al. 2007). TNF-α, which is expressed in response to LPS, appears to be a major mediator of fetal loss (Robertson et al. 2007). Regardless the low rate of success in embryo recovery from LPS injected females, we were able to obtain embryos for the embryonic time points E13,5, E15,5 and E17,5. LCN2 expression pattern in those embryos was similar to the observed in control animals. LCN2 positive cell numbers are slightly lower in the liver of embryos from LPS injected females when compared with the control counterparts. The nature of those LCN2 expressing cells is likely similar to the described above for control embryos.

The effect of the mother LPS injection in the embryo LCN2 expression is unknown. It is known that LPS administration induces LCN2 expression in post natal animals. In embryos of LPS injected females we did not observed major differences in terms of LCN2 signal distribution when comparing to the control embryos, suggesting that the protein expression was not triggered by LPS. Interestingly, there is evidence that maternally-administered LPS can induce inflammatory response in the embryo, in organs such as the liver and brain (Ning et al. 2008) (for review see Wang et al. (2006)). Whether LPS can reach the embryo itself is still unclear (Li et al. 2008). Diverse studies suggest that the inflammatory molecules that are present in embryos after maternal LPS administration are transferred from the mother into the embryo, rather than being produced by the embryo itself (Ning et al. 2008; Wang et al. 2006). Given all this data, it would be plausible to expect that maternal LPS injection would induce a visible increased LCN2 presence in the embryo. However, as reported herein, that was not the case.

We also report that embryos from LPS injected females presented slightly lower LCN2 positive cell counts in the liver, when compared with the liver of control embryos. As discussed above, it is likely that some of those LCN2 expressing cells are of the hematopoietic type. If we assume that 1) some of those LCN2 expressing cells are indeed

hematopoietic, 2) LCN2 that is expressed by the mother in response to LPS might reach the embryo and 3) LCN2 can exert pro apoptotic effects in some hematopoietic lineages in the fetal liver, identical to what happens in other hematopoietic sites (Miharada et al. 2005; Miharada et al. 2008), it is then tempting to hypothesize that this additional LCN2 (resultant from the LPS administration) might decrease the proliferation of some hematopoietic lineages in the fetal liver, thus resulting in lower numbers of putative hematopoietic LCN2 expressing cells.

Clearly more research is required in order to understand the role of LCN2 in the embryo in the context of progenitor inflammatory scenarios. Further work is also required in order to identify with more accuracy the cell types that express LCN2 in the liver of developing and newborn mice. Without this description, the biological significance of these findings remains highly elusive.

4.2. LCN2 in the post-natal liver

As referred above, in control (saline injected) post-natal animals LCN2 expression in the liver decreased in the first week of the newborn life. From P16 onwards, LCN2 positive cells were rarely seen in the liver and they normally exhibited neutrophil like morphology. On the other hand, 24 hours after the intra-peritoneal injection of LPS in post-natal mice a strong LCN2 immunostaining occurred in the liver, in an expression pattern identical between all time points analyzed. As referred above, LPS is a well known inflammatory inducer. The endotoxin recognition by mammalian cells occurs mainly by the cell surface tool-like receptor 4 (TLR4). The interaction between LPS and TLR4 results in the activation of the receptor which triggers signaling cascades in the cells that ultimately promotes the activation of immune response against the inflammatory stimulus (Freudenberg et al. 2008). While this TLR4 mediated response to inflammatory signals has been mainly associated and described in immune cells such as granulocytes and macrophages, growing evidence suggests that tissue cells, hepatocytes included, can also react to inflammatory stimulus through TLR4 mediated pathways (Liu et al. 2002; Parker et al. 2007).

LCN2 synthesis in response to inflammatory stimulus (including LPS), has been described (Cowland et al. 2003; Flo et al. 2004; Marques et al. 2008; Sommer et al. 2009;

Sunil et al. 2007). We report herein that in LPS injected post-natal animals the majority of the hepatocytes are LCN2 positive, a result consistent with previous reports where hepatic LCN2 expression was ubiquitously detected after an acute inflammatory stimulus (Flo et al. 2004). In fact, hepatocytes are responsible for the synthesis of many of the acute phase proteins that regulate inflammatory responses to infection or tissue injury (Tilg et al. 1997). LCN2 expression in mice liver after LPS administration has been reported as time-dependent, with the peak synthesis occurring between 4 and 24 hours post-injection, with the protein expression returning to basal levels 48 hours after endotoxins injection (Marques et al. 2008; Sunil et al. 2007). In addition to hepatocytes, we also observed a significant presence of neutrophil like cells (with the characteristic multilobulated shaped nuclei) stained for LCN2 in the liver of LPS injected mice of all of the studied time points. The increased presence of neutrophils in the liver after LPS injection is not surprising as tissue neutrophil mobilization and infiltration is one of the key features of mammalian immune response to inflammatory stimulus (Nussler et al. 1999).

As suggested by previous reports (Meheus et al. 1993; Sunil et al. 2007; Zhang et al. 2008) we expected to find LCN2 staining in the hepatic resident macrophages, also known as Kupffer cells, in animals that were injected with LPS. However, we did not found LCN2 positive Kupffer cells in any of the analyzed post-natal animals. Kupffer cells are pivotal to the innate immune response to acute endotoxemia, as they eliminate gram-negative pathogens and LPS from the liver and systemic circulation (Ojaniemi et al. 2006). They are also responsible for the production of inflammatory mediators such as IL-1, IL-6 and TNF-α, as well chemokines that potentiate the recruitment of inflammatory cells to the infected organ (Ramaiah and Jaeschke 2007). To date the demonstration of LPS-induced expression of LCN2 in liver macrophages is restricted to the study of Sunil et al. (2007). These authors reported LCN2 mRNA and protein expression in Kupffer cell extracts isolated from the liver of rats treated with LPS. These results contrast with our observations. We cannot discard the possibility that LCN2 expression in Kupffer cells is not strong enough to be detected by the immunohistochemistry technique we used. On the other hand it must be noticed that the Kupffer cells isolation protocol used by (Sunil et al. 2007) did not result in completely pure cellular extracts (purity above 90%), therefore the possibility of LCN2 “contamination” by cells such as hepatocytes and neutrophils in the expression results provided by Sunil et al. (2007) must not be completely discarded.

The precise mechanism(s) by which LCN2 is modulated in the liver in response to inflammation are not fully understood. LPS administration stimulates synthesis of diverse cytokines. Some cytokines, such as IL-1β and IL-1α has been reported to induce LCN2 expression in different cell types (Bando et al. 2007; Cowland et al. 2006). However, whether if such cytokines are able to elicit LCN2 synthesis at the hepatocyte level remains, to the best of our knowledge, unknown. On the other hand, there is evidence that LCN2 induction by LPS in different tissues (liver included) is TLR4 mediated (Flo et al. 2004; Sunil et al. 2007), thus indicating that a direct interaction between the pathogens and TLR4 may be required to trigger LCN2 synthesis in those tissues.

Also unclear is the biological relevance of this ubiquitous response to LPS by the liver. Some authors hypothesize that the synthesized LCN2 may down regulate inflammatory signals to prevent extended hepatic damage originated from the inflammatory response (Sunil et al. 2007). Alternatively, or in addition, LCN2 appears to contribute to the organism immune response through a bacteriostatic mechanism. As previously noticed, it has been reported that the liver synthesizes LCN2 shortly after an endotoxic stimulus. Serum levels of LCN2 are also greatly up-regulated (Flo et al. 2004; Sunil et al. 2007). LCN2 was found to compete with bacteria for the organism iron resources, thus exerting a bacteriostatic effect (Berger et al. 2006; Flo et al. 2004). Indeed, these authors demonstrated that bacteria-induced liver burden and mortality rates are higher in LCN2 knockout mice than in wild type counterparts. This bacteriostatic capacity of the protein strongly suggests that LCN2 contributes to the innate immune response through an iron depletion mechanism that prevents the proliferation of invading pathogens (Berger et al. 2006; Flo et al. 2004). Being the liver a main site of LCN2 induction in inflammatory scenarios (Flo et al. 2004), it appears possible that this hepatic produced LCN2 plays a key role in the organism response to peripheral inflammation.

Considering that our studies demonstrate a consistent pattern of LCN2 expression in the liver of LPS injected mice through all of the post natal time points, it is likely that the molecular mechanisms by which LCN2 is induced in this organ and the role of the protein in the acute immune response (and other functions as well) may be similar during the animal lifetime.

4.3. LCN2 in the developing kidney

Our study demonstrate that in addition to the liver, LCN2 positive immunoreactivity is present in the kidney of late embryos and newborn animals, an observation that suggests that the protein may be involved in the development of this organ.

The organogenesis of the kidney is a relatively complex but well described process. The formation of the definitive nephron depends on inductive interactions between two different tissue precursors, the ureteric bud (UB) that forms the collecting ducts, and the metanephric mesenchyme (MM), which differentiates into the glomeruli and renal tubules. In mouse, the UB elongates and penetrates into the MM at around E10,5. The UB then branches and MM cells aggregate at the UB tips. Afterwards, these cells undergo mesenchyme to epithelial conversion, forming renal vesicles, while the remaining mesenchyma cells form the interstitial stroma. Two consecutive clefts transform the renal vesicle into an s-shaped body. The distal end of this structure later fuses with the UB to form a continuous epithelial tubule. The differentiation of the glomerular structures occurs in the proximal end of the s-shaped body after the invasion of the proximal cleft by endothelial cells. The nephron differentiation occurs in a centrifugal fashion, with the oldest nephron localized closer to the medulla and the immature ones being present in the nephrogenic zone (NZ) in the outer cortex of the organ. UB ramification and nephron maturation in the NZ ceases in the first days of the animal life (for review see Shaw et al. (2009) and Dressler (2006))

The participation of LCN2 in the kidney development was first described by Yang et al. (2002). These authors demonstrated that LCN2 is one of the factors produced by UB cells that promote the conversion of immature MM into renal epithelial cells. The data presented suggested that this inductive action of LCN2 may be resultant of the delivery of iron by the protein into the early epithelial progenitors (Yang et al. 2002). Furthermore, they also reported *in vivo* LCN2 expression in rat UB, but not in mesenchymal cells. In addition to the mesenchyma-to-epithelia inductive effect, *in vitro* studies indicate that LCN2 also participate in the structural organization of the renal epithelia (Gwira et al. 2005). In contrast with the findings of Yang et al. (2002), we did not found LCN2 signal in the UB cells of any of the embryonic and newborn kidneys. The utilization of different rodent species and different methodological approaches may explain this discrepancy.

However, we detected LCN2 immunoreactivity in structures morphologically similar to PT in perinatal control animals. The LCN2 staining was strongly present in the cortical area of these animals until P7. The biological significance of these findings is not clear. Our observations suggest that LCN2 could be locally expressed by the proximal tubular epithelia and/or captured from the same tubules after glomerular filtration. Importantly, most of the existing data, which is resultant from studies with mature kidneys, indicates that rather from being locally expressed, LCN2 in the PT results from the rescue of the protein from the glomerular filtrate (Kuwabara et al. 2009; Mori et al. 2005; Schmidt-Ott et al. 2006; Schmidt-Ott et al. 2007). Furthermore, the protein was found to deliver iron into the PT (Mori et al. 2005; Schmidt-Ott et al. 2007). We will approach this LCN2 mediated delivery of iron in the kidney with further detail later in the discussion. Whether this recapture of LCN2 from the glomerular filtrate and consequent deliver of iron to the PT cells occurs in the perinatal kidney is currently unknown. However, since the blood of these animals contains a significant amount of non-transferrin-bound iron (NTBI) (Yang et al. 2003), it is tempting to hypothesize that LCN2 may in fact represent a pathway of relevance in the recapture and delivery of iron into the PT of the perinatal animals. As transferrin bound iron becomes the predominant form of circulating iron in older subjects (Smith and Thévenod 2009), LCN2 may lose importance to other iron related proteins such as transferrin in kidney iron reabsorption. To further clarify if LCN2 is locally synthesized or captured from the circulating pool of the protein, it would be of interest to assess the LCN2 mRNA content in the PT of the perinatal animals by *in situ* hybridization, for instance. Also, it would be of relevance to determine the PT expression levels of the putative LCN2 receptors, 24p3R and megalin. This clarification of the LCN2 origin could partially unveil the putative function of the protein in the setting of the perinatal kidney. Hence, if the protein is captured by the PT after glomeruli filtration, presumably delivering iron into the cells, one may consider that LCN2 contributes for the maintenance of the PT cell iron pool. In addition of being used in PT cells metabolic processes, this iron could also be exported into the bloodstream, either by FPN or by LCN2 itself (Schmidt-Ott et al. 2007; Devireddy et al. 2005). If, on the other hand, LCN2 is being produced by the PT cells, it is plausible to hypothesize that if the protein is secreted to the extra-cellular space, it could promote the nephron differentiation effects described above. The possibility of LCN2 recovery in the PT cells and consequent export of the protein to the kidney

parenchyma, where it could exert the referred nephron differentiation effects cannot be ruled out. Clearly further work is necessary in order to understand the origin and biological importance of LCN2 in the developing kidney.

4.4. LCN2 in the post-natal kidney

We also report here that from P16 onwards a weak LCN2 staining is present in the kidney of control animals, namely in the apical side of some PT structures. There is some contradictory data regarding the basal LCN2 expression profile in this organ. In rat, Friedl et al. (1999) report LCN2 expression in the distal portions of the organ, whereas Rached et al. (2008) depict it at the renal PT. Roudkenar et al. (2009) did not found any LCN2 signal in the organ of normal animals. In human normal kidneys, LCN2 immunoreactivity was observed by Friedl et al. (1999) and Ding et al. (2007) in the PT of the organ. On the other hand, Mori et al. (2005) demonstrates that LCN2 signal is instead present in the distal tubules and in the medullar collecting ducts of human kidney. Mishra et al. (2003) reported weak LCN2 expression in the kidney of normal mouse, an observation that is concomitant with the results herein reported for saline animals from P16 onwards.

Regardless the inconsistency of the existing data in terms of LCN2 expression in the kidney of normal subjects, the biological relevance of the protein in this context remains unclear. The work of Mori et al. (2005) was essential for a better understanding of the potential role of LCN2 in the kidney. These authors demonstrated *in vivo* that tagged LCN2 is quickly recaptured by the PT of the nephron after glomerular filtration. Furthermore, through injection with ^{55}Fe-loaded LCN2 they proved that the protein can deliver iron into PT cells (Mori et al. 2005). The authors also propose that the endogenous LCN2 may exert identical functions. It is then plausible to consider that the herein reported LCN2 presence in the kidney of control animals from P16 onwards might be resultant from the protein recapture from the glomerular filtrate. Furthermore, the protein staining in these kidney samples was very faint, suggesting low rates of the protein uptake from the filtrate. This observation is concomitant with the low levels of serum LCN2 in healthy individuals (Jiang et al. 2008; Flo et al. 2004; Mori et al. 2005).

Iron recovery by the kidney from the glomerular filtrate appears to rely on multiple pathways, with the most well described ones being mediated by DMT1 and transferrin

(Smith and Thévenod 2009). The current data indicate that despite the apparent limited relevance, LCN2 may constitute another mechanism of iron recuperation from the glomerular filtrate, thus contributing to the body iron homeostasis; the extent of LCN2 participation in these processes needs to be further clarified. Interestingly, the iron delivery capabilities of LCN2 in the kidney appear to gain special importance in kidney disease conditions, as it will be next discussed.

Etiologies such as kidney transplantation and cardiac cirurgy can result in acute kidney injury (AKI) (Di Grande et al. 2009). Sepsis can also result in AKI. Indeed, depending on the septic severity, up to 51% of septic patients present AKI. Furthermore, the mortality rates are higher in septic patients that develop AKI than in patients with AKI alone (Schrier and Wang 2004). As an model of gram-negative sepsis, LPS administration is capable of inducing renal dysfunction and injury (Cunningham et al. 2002; Cunningham et al. 2004; Guo et al. 2004; Heemskerk et al. 2006; Langenberg et al. 2008; Yura et al. 2009). The pathophysiology of sepsis-induced AKI is complex and far from fully understood (Wan et al. 2008). Nevertheless, an increasing body of evidence indicates that diverse mechanisms may participate in this condition. These include alterations in systemic and renal hemodynamics, renal cell damaging mediated by systemic and/or locally produced inflammatory cytokines such as TNF-α and increased renal oxidative stress (Wan et al. 2008; Wang et al. 2005; Wu et al. 2007; Zager et al. 2006; Zhang et al. 2000). Diverse studies report that increased levels of LCN2 in the serum and urine observed after diverse etiologies such as renal transplant and sepsis correlates significantly with the development of kidney damage (Mishra et al. 2006; Mori et al. 2005; Wheeler et al. 2008). This up-regulation of the protein is strong and fast, supporting LCN2 as a sensible and predictive biomarker of AKI (Di Grande et al. 2009).

We report herein that the intraperitoneal administration of LPS resulted in a strong LCN2 signal in the kidney of all post-natal animals. In all time points, a significant portion of the cortical PT was stained for LCN2. Interestingly, our results are consistent with studies that report renal LCN2 expression in diverse AKI scenarios (Devarajan 2008; Kuwabara et al. 2009; Mishra et al. 2006; Mori et al. 2005). It must be noticed that we did not evaluated the damage levels of the renal structures in any sample. The serum levels of LCN2 rise greatly in inflammatory situations (Flo et al. 2004; Marques et al. 2008), with the liver presumably being one of the main sources of the protein (Flo et al. 2004).

Interestingly, much of the observed LCN2 staining in the kidney PT was concentrated in the apical side of the tubular structure, suggesting that the protein may be recaptured in the PT. This observation is consistent with previous reports that demonstrate that LCN2 can be reabsorbed by PT cells from the glomerular filtrate (Kuwabara et al. 2009; Mori et al. 2005; Schmidt-Ott et al. 2007). As previously discussed, exogenous LCN2 can also deliver iron into the PT cells, as reported by Mori et al. (2005). These authors further demonstrated that this delivery of iron-loaded LCN2 leads to up regulation of Hox1 and maintenance of N-cadherin, both proteins that favor cellular viability (Mori et al. 2005). Indeed, administration of LCN2 before or after ischemic-injury can minimize kidney damage (Mishra et al. 2004; Mori et al. 2005). It is therefore possible that if the observed LCN2 in the PT of LPS injected animals results from glomerular filtrate recapture, the protein may be contributing for the maintenance of cellular viability of the tubular structures, thus protecting the renal tubules from possible damage.

In addition to the LCN2 staining observed in the PT, we also report that LPS injection results in LCN2 immunoreactivity in the kidney DT and collecting ducts. Interestingly, this pattern of immunoreactivity was only visible from two month old animals onwards, since in younger animals neither DT nor collecting ducts presented the protein signal. The observed staining in the distal portion of the nephron in older animals is consistent with the reported increase of the protein in the same structures in AKI scenarios of rodent and humans (Kuwabara et al. 2009; Mishra et al. 2006; Schmidt-Ott et al. 2006). Evidence indicates that the LCN2 synthesized in the distal sections of the kidney in AKI settings is released into the urine (Schmidt-Ott et al. 2007). It has been hypothesized that this secreted LCN2 could promote an anti-bacterial effect in the lower urinary tract (Schmidt-Ott et al. 2007). In addition, as the distal segments are also damaged in AKI, some authors consider that the expressed LCN2 may also favor the survival of the distal cells, although no clear evidence supporting this hypothesis exists (Devarajan 2008; Devarajan 2010).

The mechanism of LCN2 induction in the distal nephron by LPS is currently unclear. As part of the innate immune response to LPS, the systemic levels of inflammatory mediators such as TNF-α and IL-1β are highly increased after the endotoxin recognition by systemic TLR4 (Cohen 2002; El-Achkar et al. 2008; Ramesh et al. 2007; Tracz et al. 2007). Since IL-1β can induce LCN2 synthesis in epithelial cell lines (Cowland

et al. 2006), it is plausible to speculate that LCN2 expression in kidney tubules may be induced by such mediators. Interestingly, recent studies report that a direct endotoxin-kidney cells interaction may also contribute to the renal response to inflammatory stimulus (Cunningham et al. 2004; El-Achkar et al. 2008). Indeed, TLR4 is found present in most of the rat kidney, with both mRNA and protein expression being reported in the distal portion of the nephron (El-Achkar et al. 2008). Similarly, TLR4 mRNA is found present in the distal nephron of mice (Wolfs et al. 2002). Kidney TLR4 expression increases after septic stimulus (El-Achkar et al. 2006). Furthermore, injected endotoxin can access the renal tubules, including sections of the distal nephron (El-Achkar et al. 2006). Taking these data in consideration, we might consider that the observed LCN2 signal in the DT and medulla may be a consequence, at least partially, of local TLR4 activation by LPS. It is noteworthy that while PT cells present TLR4 and endotoxin can access those cells (El-Achkar et al. 2008), there is not, to the best of our knowledge, evidence that LCN2 expression is triggered in these cells. Instead, the protein presence in the PT cells appears to be correlated with absorption of the protein from the glomerular filtrate, as described above.

In addition to the action of inflammatory mediators and/or endotoxin stimulus, it is possible that the herein described presence of LCN2 in the distal nephron occurs in response to a possible undergoing epithelial stress and/or damage. Indeed, LCN2 expression is significantly induced in damaged epithelial cells of organs such as liver, lung and colon (Devarajan 2008; Devarajan 2010; Wheeler et al. 2008). Concomitantly, LCN2 is expressed by damaged renal epithelia in diverse renal diseases and conditions (Kuwabara et al. 2009). However, it must be noticed that while LPS has been reported to induce AKI (as discussed before), most of the histological findings describe damage only in the proximal components of the nephron. Indeed, to the best of our knowledge, there is no histological data demonstrating LPS-induced damage in the distal nephron.

We report herein that LPS administration results in an evident increase of LCN2 expression in the kidney of the analyzed post-natal animals. Interestingly, the pattern of the renal LCN2 distribution was not homogenous trough age. Indeed, we observed a lack of LCN2 signal in the distal portion of the nephron in young mice injected with LPS, with the protein immunoreactivity, which first appears in this renal area in the two month old animals, being ubiquitously present in the DT and medulla of older animals. The cellular and/or molecular mechanisms behind this striking difference and corresponding biological

relevance are unknown. Some reports suggest that kidney susceptibility to diverse kidney-damaging substances, endotoxin included, appears to be dependent of the age/developmental phase of the organ, with younger kidneys being less affected by the damaging stimulus than older counterparts. Various factors may be accountable for these age-related differences. Of special importance appears to be the functional maturity of the kidney. It is known that the kidney morphological differentiation achieves completion shortly after birth. At this period, however, the organ is far from being fully functional. In rodents, the functional maturation of the kidney develops quickly in the post-natal period. The levels of various renal transporters increase significantly in the first weeks of the animal life (Suzuki 2009). Concomitantly, the renal blood flow (RBF) and glomerular filtration rate (GFR) are found to increase in the first weeks of life in the rat (Horster 1977; Solhaug et al. 2004; Suzuki 2009). Some authors suggest that this reduced RBF and GFR observed in younger animals may result in reduced renal damage in AKI scenarios (Solhaug et al. 2004). Indeed, low GFR is thought to be behind the lower glomerular changes observed in younger rats injected with gram-negative *Salmonella enteritidis* when compared with older counterparts (Hurley et al. 1989; Solhaug et al. 2004). Lower RBF in younger animals is accountable for the reduced aminoglycosides-induced renal damage when compared with adult animals (Cowan et al. 1980; Solhaug et al. 2004). Concomitant with these findings, cisplatin-induced AKI is more evident in older rodents than in young ones (Ali et al. 2008; Appenroth et al. 1988; Espandiari et al. 2009; Fleck et al. 2001).

In addition to kidney hemodynamics, other factors may contribute for the reduced levels of renal damage in younger animals. For instance, Zager et al. (2008) observed that as LPS-induced AKI increases with age, the basal levels of renal cortical cholesterol, a molecule that exerts protective effects in the kidney cells, actually decreases through the same period, thus indicating that cholesterol may be one of the contributing factors for the different age-related sensibilities of the kidney to the endotoxin effects. The authors further hypothesize that age-dependent fluctuation in the expression of other molecules such as growth factors and Hox1 may also contribute for this effect (Zager et al. 2008). Taking all this data in consideration, it appears evident that the kidney response to some damaging stimulus is dependent of the developmental stage of the organ and that multiple mechanisms may be behind this phenomenon. Considering that LCN2 signal in the distal portion of the kidney appears to reflect underlying cell damage (Kuwabara et al. 2009), it

is tempting to speculate that the herein reported lack of LCN2 signal in the distal nephron of young mice might reflect reduced/ absent damage in those cells. This (putative) reduced damage in the kidney of younger animals might be attributed to the factors discussed above.

In summary, our results demonstrate that an acute inflammatory stimulus induces a strong LCN2 presence in mice kidney tubules. The distribution of the protein signal was age-dependent, with the kidney of older animals displaying LCN2 immunoreactivity trough all of the nephron. Whereas the biological significance of this expression pattern is unclear, it is possible that it is linked to acute renal damage. LCN2, possibly trough iron delivery, may promote the survival of renal PT cells, thus limiting renal damage. Furthermore, LCN2 expression by the distal nephron (which presumably occurs in response to local epithelial damage) might exert a bacteriostatic effect in the lower urinary tract. The absence of LCN2 signal in the distal nephron of younger animals might reflect a lower susceptibility of those structures to renal damage. It would be of interest to assess the histological damage in the various portions of the nephron and try to correlate it with LCN2 immunoreactivity. Further work is required in order to better understand the biological significance of renal LCN2 presence in the context of acute phase response to inflammatory stimulus.

4.5. LCN2 in the brain

LCN2 presence in the embryonary brains, either from saline or LPS injected progenitors, was limited to occasional staining in the stromal space of the choroid plexus (CP). Identical staining pattern was observed in newborn (P3) saline injected animals. These LCN2-expressing cells are likely to be circulating cells such as neutrophils. In older saline injected animals no LCN2 expression was visible in the brain. On the other hand, LPS peripheral administration in post natal animals resulted in evident LCN2 expression in different areas of the brain, namely in the CP epithelia and stroma, ependyma and endothelial cells of the blood vessels. This pattern was consistent in all post-natal animals injected with LPS with the notable exception of 3 day-old pups, were no LCN2 signal was observed in the epithelial cells of the CP, despite the protein expression in the CP stroma,

ependyma and blood vessels. The CP consists of a network of capillaries surrounded by a monolayer of epithelial cells that is located within the brain ventricles, being the epithelial layer responsible for the production of most of the cerebrospinal fluid (CSF) present in the brain (Speake et al. 2001). Whereas CP capillaries are fenestrated, the epithelial cells junctions are tight, thus limiting the passage of substances and cells through the cell layer into the CSF. For this reason, CP is part of the blood-CSF barrier (Redzic and Segal 2004). In addition to CSF synthesis and restriction of molecular/cellular passage from blood to CSF, the CP produces molecules that are important for the regular CNS functioning, such as growth factors and hormones (Skipor and Thiery 2008). Interestingly, the CP also has receptors for most of these molecules, suggesting an autocrine and/or paracrine modulation of CP functioning (Chodobski and Szmydynger-Chodobska 2001).

Increasing evidence suggests that the CP may have an important role in the immune response to inflammatory stimulus in the CNS. For instance, the main receptor of LPS, TLR4, is expressed in physiological conditions in rodents CP (Laflamme and Rivest 2001; Chakravarty and Herkenham 2005). Furthermore, inflammatory mediators such as TNFα, IL-1 and IL-6 were found up-regulated after inflammatory stimulus in the CP epithelial cells and CSF (Chakravarty and Herkenham 2005; Marques et al. 2007; Marques et al. 2009; Nadeau and Rivest 1999; Quan et al. 1999). Marques et al also reported higher levels of LCN2 expression in the CP and CSF of LPS challenged mice (Marques et al. 2008). In accordance with this study by Marques et al, we demonstrate here that LPS administration clearly induced LCN2 immunostaining in the CP epithelial cells, stromal cells and blood vessels of the studied post-natal time points, with the exception of P3 old animals.

The mechanisms behind the induction of LCN2 synthesis in the CP by LPS are unclear. Identical to what we discussed before, it is possible that certain cytokines may trigger LCN2 synthesis in the CP after an inflammatory stimulus. Concomitantly, IL-1β receptor is expressed by the CP (Cunningham et al. 1992). It is also possible that LPS induced LCN2 expression in the CP is TLR4 mediated (Marques et al. 2008). The herein reported absence of LCN2 expression in the CP epithelia of LPS injected P3 animals suggests an impaired response of the CP in terms of LCN2 synthesis to the peripheral inflammatory stimulus in those animals. The reason for this apparent lack of response to LPS is unclear. It is known that newborns are more susceptible to infections, presumably due to incomplete maturation of both adaptative and innate immune systems (Belderbos et

al. 2009). Interestingly, diverse studies report that TLR mediated response to inflammatory stimulus (such as LPS) is impaired in blood cells of neonatal subjects, with the expression levels of cytokines such as TNF- α, IL-6 and interleukin-12 being lower in newborn blood cells that were treated with LPS, when compared with older counterparts (Belderbos et al. 2009; Chelvarajan et al. 2004; Levy et al. 2004; Wit et al. 2003). Concomitantly, TLR4 mediated activation of NF-kB, a key transcriptional factor for expression of cytokines, is found depressed in neonatal monocytes and macrophages (Maródi 2006). Maródi suggests that this apparent impairment of TLR signaling pathways and consequent diminution of the inflammatory response may be, at least partially, responsible for the deficient innate immunity of newborns. Given these data, it may be hypothesized that the lack of LCN2 expression in the CP epithelia of P3 animals injected with LPS might be a result of impairment of TLR4 signaling. Furthermore, it must be noticed that TLR4 expression itself can be tissue and age dependent (Harju et al. 2001). The profiling of TLR4 expression in the CP of newborn animals has not been reported, despite the description of TLR4 mRNA presence in the brain of 7 days old rats (Eklind et al. 2001). In addition, the sensitivity to LPS can be dependent of the levels of TRL4 expression (Bihl et al. 2003). Therefore, it is plausible to hypothesize that the absent LCN2 expression in the CP epithelia of P3 animals may be a consequence of: 1) decreased functionality of TLR4 signaling and/or 2) low expression of TLR4 in the newborn CP.

Taking into account the described bacteriostatic capacity of LCN2 and the reported presence of the protein in both CP and CSF after LPS challenge, it is possible that LCN2 may be important to restrict bacterial penetration and proliferation in the CSF, thus representing a significant element of the innate immune protection of the CNS (Marques et al. 2008). Our work suggests that while this putative innate immune mechanism is present trough the animal lifespan, it may be somehow compromised in newborn animals.

Interestingly, in addition to LCN2, other iron-related genes were found altered in the CP and CSF after a peripheral inflammatory stimulus. Indeed, Marques, Falcao, et al. (2009) report an increase in the expression of genes encoding for hepcidin and ferritin in the CP shortly after LPS injection. They also observed an increase in hepcidin prohormone in the CSF. These observations led the authors to hypothesize that in response to the inflammatory stimulus, the CP may increase its iron storage capacity and at the same time reduce the export of iron into the CSF, thus promoting decreased iron availability in the

CSF (Marques, Falcao, et al. 2009). Since LCN2 is also synthesized by the CP and secreted into the CSF, it appears likely that this protein may also contribute for the alteration of the CSF iron status, thus ultimately participating in the modulation of iron homeostasis in the brain (Marques et al. 2008; Marques et al. 2009). Our results suggest that this contribution of LCN2 to the regulation of CSF iron homeostasis in acute phase scenarios may be consistent through age, with the exception of the first 3 day old animals, as we did not detected LCN2 signal in the CP epithelial cells of those animals.

We report here for the first time LCN2 presence in cells of the ependyma layer. Immunoreactivity was detected in all post-natal animals that were injected with LPS. The biological relevance of ependymal LCN2 presence is unknown. The ependyma layer lines the ventricular cavity of the central nervous system and is formed by a simple layer of epithelial cells (ependymal cells). Most of this ependymal cells present microvilli and cilia. The ependyma constitutes a barrier between CSF and brain extracellular fluid. The cilia present in ependyma cells beat in a coordinated fashion, suggesting that the layer may promote the CSF movement through the ventricles (Del Bigio 2010). Ependyma may regulate the transport of diverse substances between CSF and the brain (Bruni 1998; Hauwel et al. 2005). It is then likely that the ependyma may protect the brain from hazardous metabolites present in the CSF (Del Bigio 1995; Kuchler et al. 1994). Furthermore, ependyma also responds to inflammatory stimulus through cytokines expression (Hauwel et al. 2005; Xia et al. 2006), suggesting that it might act as a first line of defense against infection in the brain (Del Bigio 2010). This cytokine expression is possibly TLR mediated, with TLR4 being present in physiological conditions in the ependyma (Chakravarty and Herkenham 2005).In addition, the protein is upregulated after LPS challenge (Xia et al. 2006). We report here LCN2 presence in some cells of the ependyma layer of LPS injected animals. Whether LCN2 is expressed by the ependyma itself or is absorbed by the CSF is unclear. The observation that in P3 LPS injected animals LCN2 signal is present in the ependyma but not in the epithelia CP supports the LCN2 expression by the ependyma hypothesis. How LCN2 is induced in the ependyma and its biological function needs to be clarified.

Further studies are required in order to better understand the role LCN2 in cerebral immunity and iron homeostasis, in particular in newborns. It would be of interest to determine with more accuracy the onset of LPS-induced LCN2 expression in the CP. Also,

the measurement of CP TLR4 levels and associated inflammatory mediators in the newborn subjects could allow a better understanding of the molecular mechanisms responsible for the herein reported differences in terms of CP LCN2 expression between LPS injected newborn and older animals.

4.6. LCN2 in the spleen

The profile of LCN2 expression in the spleen of post-natal animals did not change significantly through age. In control animals the staining was restricted to some cells of the red pulp area of the organ. We have not identified the cell types that present LCN2 expression. Flo et al. (2004) reported that in the red pulp area of spleen of C57BL/6 control mice only neutrophils displayed LCN2 signal. It is then likely that most of the herein observed LCN2 expressing cells in that splenic area are indeed neutrophils.

LPS injection resulted in an increase in the presence of LCN2 positive cells in the spleen in all studied time points, mainly in the red pulp area. Although we did not identify the remaining populations of LCN2 expressing cells, it is likely that they are of identical nature as reported by Flo et al. (2004). These authors described an induction of LCN2 expression in red pulp macrophages, fibroblasts and endothelial cells in *E. coli* injected C57BL/6 mouse (Flo et al. 2004). Furthermore, we report here a slight increase in the occurrence of LCN2 stained cells in the white pulp of the organ, an observation similar to the reported by Flo et al. (2004). Accordingly to these authors, the LCN2 expressing cells in the white pulp presented morphologic characteristics of macrophages and dendritic cells, thus it is likely that the herein reported white pulp LCN2 positive cells are of the same type.

The biological relevance of this expression pattern is, to the best of our knowledge, unknown. It is known that in addition to the liver, the spleen is a primary site to LPS response (Smolinski and Pestka 2005). Splenic macrophages display phagocytic activity towards the endotoxin, thus contributing to its elimination from the bloodstream (Feleder and Blatteis 2006). In addition, the activation of splenic macrophages and other splenic cells such as dendritic cells by the endotoxic stimulus results in the prompt production of inflammatory mediators by those cells (Randolf et al. 2009; Smolinski and Pestka 2005; Tracey 2007). Indeed, the spleen is an important site of inflammatory cytokine synthesis

during inflammation scenarios, with the organ being the main producer of systemic TNF (Huston et al. 2006; Smolinski and Pestka 2005), further demonstrating that this organ plays a role in the immune response to inflammatory agents.

While the liver appears to be an important source of LCN2 following an inflammatory stimulus (Flo et al. 2004), the precise contribution of the spleen to the systemic levels of LCN2 in such scenarios is unknown. Given the (above discussed) contribution of the spleen to inflammatory mediators production, the response of the organ in terms of LCN2 immunoreactivity to an *E.coli* challenge and the significant increase of serum LCN2 after the bacterial stimulus (Flo et al. 2004), it is possible that the spleen can also be a source of systemic LCN2.

We report here that LPS (which is a major component of gram-negative cell wall) administration induces increased splenic LCN2 immunoreactivity in all of the post-natal animals in an identical pattern, suggesting that the mechanisms by which the protein is induced and the biological relevance may be similar through age. Further studies are necessary in order to better understand the relevance of splenic LCN2, both locally and systematically, to the organism response to an inflammatory stimulus.

4.7. LCN2 in the thymus

We report herein the presence of LCN2 stained cells in the thymus of all post-natal time points. For each analyzed age we did not found significant differences in the pattern of LCN2 expression between control and LPS animals. We observed more LCN2 stained cells in the thymus of animals ranging from P16 to P60, with the cells being identically scattered between the organ medulla and cortex. We did not identify the nature of these LCN2 expressing cells. While LCN2 presence in the thymus was previously reported (Berger et al. 2006; Cowland and Borregaard 1997; Devireddy et al. 2001; Miharada et al. 2005), the immunohistochemical description of the protein in this organ is restricted to the work of Friedl et al. (1999). These authors depict LCN2 signal in both human and rat Hassall's corpuscles, which are epithelial structures present in the thymic medulla. Furthermore, they also describe LCN2 expression in other epithelial cells of the medulla of rat thymus. It is possible that some of the herein reported signal of LCN2 in the thymus is originated by these particular structures. Interestingly, Friedl et al. (1999) do not describe

LCN2 expression in either rat or human thymic cortex, an observation that contradicts our results. Although the organ histology is found conserved between diverse species (Haley 2003), the thymic LCN2 expression pattern might be, to some extent, species-dependent.

As mentioned above, LCN2 presence in the thymus has already been described. The protein relevance in the organ functioning, however, is currently unknown. The thymus is the principal site of T-lymphocytes (T cells) formation in mammals. T cells are key mediators of adaptive immune response and their maturation from immature thymocytes is a highly regulated process that occurs in various discrete steps. Diverse molecules present in the thymic niche modulate the thymocyte maturation processes (Hale and Fink 2009; Savino et al. 2007). Interestingly, Devireddy et al. (2001) suggests that LCN2 might also participate in such processes. These researchers observed that LCN2 primary thymocytes incubated with LCN2 display higher levels of apoptosis. Furthermore, they reported that primary thymocytes incubated with dexamethasone, a synthetic glucocorticoid that is known to induce apoptosis in thymocytes (Walker et al. 1991), synthesize LCN2. These results lead to the hypothesis that LCN2 may be involved in thymocyte selection processes (Devireddy et al. 2001). In contrast with these results, Berger et al. (2006) reported that LCN2 deficiency does not affect the apoptotic profile of primary thymocytes incubated with dexamethasone. They also did not found differences in thymocyte population between LCN2 deficient and wild type mice.

We report here that thymic LCN2 is present through the mouse lifetime (at least till the age on one year), with the protein expression being more evident in the P16-P60 period, thus coinciding with the animal sexual maturation. It is known that the size and activity (T cell formation) of the organ increases until sexual maturity (Pearse 2006). The organ then gradually atrophies due to circulating sex hormones, with functional thymic tissue being replaced by fat. Consequently, the thymic T cell output also decreases with age (Savino 2000; Sutherland et al. 2005). The observation that the herein described peak of thymic LCN2 is coincident with the organ higher activity in terms of T cell formation further supports the possibility of a LCN2 participation in such processes.

The data regarding the functional aspects of thymic LCN2 is scarce and not consensual. Clearly more work is required in order to better understand the role of LCN2 in the functioning of the organ.

5

Conclusions, Future perspectives and Final Remarks

5.1. Conclusions

We report here that LCN2 presence and distribution in various organs vary with age and physiological state. We hypothesize that the observed LCN2 expression in the fetal and newborn liver is likely linked to the hematopoietic processes that are known to occur in this organ. The prominent LCN2 presence in the control perinatal kidney may reflect the involvement of the protein in the organ development, possibly through iron delivery. The overall LCN2 induction by LPS in all of the studied tissues (with the exception of the thymus) in the post-natal period suggests that the mechanisms by which the protein is induced and its biological functions may be preserved through age in each organ.

5.2. Future perspectives and final remarks

With this work we provide some previously unreported observations that ultimately raise new questions regarding LCN2 biology. Among others, it would be relevant to address the following issues in the future:

- What is the contribution of LCN2 for kidney development?
- What is the functional and biological significance of age-dependent variation in the kidney response, regarding LCN2 expression, to the LPS endotoxin?
- How does the lack of LCN2 synthesis by CP epithelia after an LPS stimulus in newborn mice impacts the overall brain response to infection and the CSF iron balance?
- Why is the LCN2 expression lower in the embryonic liver of dam from LPS injected pregnants?

Our findings further suggest that future studies regarding LCN2 relevance in certain tissues should consider the age factor. Although LCN2 biology is not fully understood, growing evidence indicates that the protein is pertinent in many aspects of mammalian life. The protein has promising clinical applications in perspective, namely in the context of kidney injury. Further research is required to define the precise functions of LCN2 and to possible unveil new clinical application for the protein.

6

References

Abouhamed, M., Gburek, J., Liu, W., Torchalski, B., Wilhelm, A., Wolff, N. a., Christensen, E. I., Thévenod, F., and Smith, C. P. (2006). "Divalent metal transporter 1 in the kidney proximal tubule is expressed in late endosomes/lysosomal membranes: implications for renal handling of protein-metal complexes." *Am J Physiol. Renal physiol*, 290(6), F1525-33.

Ali, B. H., Al-Moundhri, M., Tageldin, M., Al Husseini, I. S., Mansour, M. a., Nemmar, a., and Tanira, M. O. (2008). "Ontogenic aspects of cisplatin-induced nephrotoxicity in rats." *Food and chemical toxicology*, 46(11), 3355-9.

Alpízar-Alpízar, W., Laerum, O. D., Illemann, M., Ramírez, J. A., Arias, A., Malespín-Bendaña, W., Ramírez, V., Lund, L. R., Borregaard, N., and Nielsen, B. S. (2009). "Neutrophil gelatinase-associated lipocalin (NGAL/Lcn2) is upregulated in gastric mucosa infected with Helicobacter pylori." *Virchows Archiv*, 455(3), 225-33.

Anderson, G. J., Darshan, D., Wilkins, S. J., and Frazer, D. M. (2007). "Regulation of systemic iron homeostasis: how the body responds to changes in iron demand." *BioMetals*, 20(3-4), 665-674.

Appenroth, D., Winnefeld, K., and Bräunlich, H. (1988). "Nephrotoxicity and pharmacokinetics of cisplatinum in young and adult rats." *Biomed Biochim Acta*, 47(8), 791-7.

Bando, M., Hiroshima, Y., Kataoka, M., Shinohara, Y., Herzberg, M. C., Ross, K. F., Nagata, T., and Kido, J. (2007). "Interleukin-1alpha regulates antimicrobial peptide expression in human keratinocytes." *Immunol Cell Biol*, 85(7), 532-7.

Belderbos, M. E., van Bleek, G. M., Levy, O., Blanken, M. O., Houben, M. L., Schuijff, L., Kimpen, J. L., and Bont, L. (2009). "Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life." *Clin Immunol ,* 133(2), 228-37.

Berger, T., Togawa, A., Duncan, G. S., Elia, A. J., You-Ten, A., Wakeham, A., Fong, H. E., Cheung, C. C., and Mak, T. W. (2006). "Lipocalin 2-deficient mice exhibit increased sensitivity to Escherichia coli infection but not to ischemia-reperfusion injury." *Proc Natl Acad Sci USA*, 103(6), 1834-9.

Bihl, F., Salez, L., Beaubier, M., Torres, D., Larivière, L., Laroche, L., Benedetto, A., Martel, D., Lapointe, J., Ryffel, B., and Malo, D. (2003). "Overexpression of Toll-like receptor 4 amplifies the host response to lipopolysaccharide and provides a survival advantage in transgenic mice." *J Immunol*, 170(12), 6141-50.

Bolignano, D., Coppolino, G., Campo, S., Aloisi, C., Nicocia, G., Frisina, N., and Buemi, M. (2008). "Urinary neutrophil gelatinase-associated lipocalin (NGAL) is

associated with severity of renal disease in proteinuric patients." *Nephrol Dial Transplant*, 23(1), 414-6.

Breuer, W., Shvartsman, M., and Cabantchik, Z. I. (2008). "Intracellular labile iron." *Int J Biochem Cell Biol.*, 40(3), 350-4.

Bruni, J. E. (1998). "Ependymal development, proliferation, and functions: a review." *Microsc Res Tech*, 41(1), 2-13.

Burne, M. J., Osicka, T. M., and Comper, W. D. (1999). "Fractional clearance of high molecular weight proteins in conscious rats using a continuous infusion method." *Kidney Int*, 55(1), 261-70.

Canonne-Hergaux, F., and Gros, P. (2002). "Expression of the iron transporter DMT1 in kidney from normal and anemic mk mice." *Kidney Int* 62(1), 147-56.

Chakravarty, S., and Herkenham, M. (2005). "Toll-like receptor 4 on nonhematopoietic cells sustains CNS inflammation during endotoxemia, independent of systemic cytokines." *J. Neurosci.*, 25, 1788-1796.

Chelvarajan, R. L., Collins, S. M., Doubinskaia, I. E., Goes, S., and Willigen, J. V. (2004). "Defective macrophage function in neonates and its impact on unresponsiveness of neonates to polysaccharide antigens." *J Leukoc Biol*, 10 -13.

Chodobski, a., and Szmydynger-Chodobska, J. (2001). "Choroid plexus: target for polypeptides and site of their synthesis." *Microsc Res Tech*, 52(1), 65-82.

Chua, A. C., Graham, R. M., Trinder, D., and Olynyk, J. K. (2007). "The regulation of cellular iron metabolism." *Crit Rev Clin Lab Sci*, 44(5-6), 413-59.

Cohen, J. (2002). "The immunopathogenesis of sepsis." *Nature*, 420(6917), 885-91.

Cowan, R. H., Jukkola, A. F., and Arant, B. S. (1980). "Pathophysiologic evidence of gentamicin nephrotoxicity in neonatal puppies." *Pediatric res*, 14(11), 1204-11.

Cowland, J. B., Muta, T., and Borregaard, N. (2006). "IL-1beta-specific up-regulation of neutrophil gelatinase-associated lipocalin is controlled by IkappaB-zeta." *J Immunol*, 176(9), 5559-66.

Cowland, J. B., Sørensen, O. E., Sehested, M., and Borregaard, N. (2003). "Neutrophil gelatinase-associated lipocalin is up-regulated in human epithelial cells by IL-1 beta, but not by TNF-alpha." *J Immunol*, 171(12), 6630-9.

Cowland, J. B., and Borregaard, N. (1997). "Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans." *Genomics*, 45(1), 17-23.

Crichton, R. R., Wilmet, S., Legssyer, R., and Ward, R. J. (2002). "Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells." *J Inorg Biochem*, 91(1), 9-18.

Cumano, A., and Godin, I. (2007). "Ontogeny of the hematopoietic system." *Annu Rev Immunol*, 25, 745-85.

Cunningham, E. T., Wada, E., Carter, D. B., Tracey, D. E., Battey, J. F., and De Souza, E. B. (1992). "In situ histochemical localization of type I interleukin-1 receptor messenger RNA in the central nervous system, pituitary, and adrenal gland of the mouse." *J neurosci*, 12(3), 1101-14.

Cunningham, P. N., Dyanov, H. M., Park, P., Wang, J., Newell, K. A., and Quigg, R. J. (2002). "Acute renal failure in endotoxemia is caused by TNF acting directly on TNF receptor-1 in kidney." *J Immunol*, 168(11), 5817-23.

Cunningham, P. N., Wang, Y., Guo, R., He, G., and Quigg, R. J. (2004). "Role of Toll-like receptor 4 in endotoxin-induced acute renal failure." *J Immunol*, 172(4), 2629-35.

De Domenico, I., Ward, D. M., and Kaplan, J. (2007). "Hepcidin regulation: ironing out the details." *J Clin Invest*, 117(7), 1755-8.

Del Bigio, M. R. (1995). "The ependyma: a protective barrier between brain and cerebrospinal fluid." *Glia*, 14(1), 1-13.

Del Bigio, M. R. (2010). "Ependymal cells: biology and pathology." *Acta neuropathol*, 119(1), 55-73.

Devarajan, P. (2008). "Emerging urinary biomarkers in the diagnosis of acute kidney injury." *Expert Opin Med Diagn*, 2(4), 387-398.

Devarajan, P. (2008). "Neutrophil gelatinase-associated lipocalin (NGAL): a new marker of kidney disease." *Scand J Clin Lab Invest Suppl*, 241, 89-94.

Devarajan, P. (2008). "Neutrophil gelatinase-associated lipocalin--an emerging troponin for kidney injury." *Nephrol Dial Transplant*, 23(12), 3737-43.

Devarajan, P. (2010). "Review: neutrophil gelatinase-associated lipocalin: a troponin-like biomarker for human acute kidney injury." *Nephrology (Carlton)*, 15(4), 419-28.

Devireddy, L. R., Gazin, C., Zhu, X., and Green, M. R. (2005). "A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake." *Cell*, 123(7), 1293-305.

Devireddy, L. R., Teodoro, J. G., Richard, F. A., and Green, M. R. (2001). "Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation." *Science*, 293(5531), 829-34.

Di Grande, A., Giuffrida, C., Carpinteri, G., Narbone, G., Pirrone, G., Di Mauro, A., Calandra, S., Noto, P., Le Moli, C., Alongi, B., and Nigro, F. (2009). "Neutrophil gelatinase-associated lipocalin: a novel biomarker for the early diagnosis of acute kidney injury in the emergency department." *Eur Rev Med Pharmacol Sci*, 13(3), 197-200.

Ding, H., He, Y., Li, K., Yang, J., Li, X., Lu, R., and Gao, W. (2007). "Urinary neutrophil gelatinase-associated lipocalin (NGAL) is an early biomarker for renal tubulointerstitial injury in IgA nephropathy." *Clin Immunol*, 123(2), 227-34.

Dressler, G. R. (2006). "The cellular basis of kidney development." *Annu Rev Cell Dev Biol*, 22, 509-29.

Dunn, L. L., Rahmanto, Y. S., and Richardson, D. R. (2007). "Iron uptake and metabolism in the new millennium." *Trends Cell Biol*, 17(2), 93-100.

Eklind, S., Mallard, C., Leverin, A., Gilland, E., Blomgren, K., Mattsby-baltzer, I., and Hagberg, H. (2001). "Bacterial endotoxin sensitizes the immature brain to hypoxic--ischaemic injury." *Eur J Neurosci*, 13, 1101-1106.

El-Achkar, T. M., Hosein, M., and Dagher, P. C. (2008). "Pathways of renal injury in systemic gram-negative sepsis." *Eur J Clin Invest*, 38 Suppl 2, 39-44.

El-Achkar, T. M., Huang, X., Plotkin, Z., Sandoval, R. M., Rhodes, G. J., and Dagher, P. C. (2006). "Sepsis induces changes in the expression and distribution of Toll-like receptor 4 in the rat kidney." *Am J Physiol Renal Physiol*, 290(5), F1034-43.

Ema, H., and Nakauchi, H. (2000). "Expansion of hematopoietic stem cells in the developing liver of a mouse embryo." *Blood*, 95(7), 2284-8.

Espandiari, P., Rosenzweig, B., Zhang, J., Zhou, Y., Schnackenberg, L., Vaidya, V. S., Goering, P. L., Brown, R. P., Bonventre, J. V., Mahjoob, K., Holland, R. D., Beger, R. D., Thompson, K., Hanig, J., and Sadrieh, N. (2009). "Age-related differences in susceptibility to cisplatin-induced renal toxicity." *J Appl Toxicol*, 30(2), 172-82.

Feleder, C., and Blatteis, C. (2006). "The role of the spleen in the febrile response induced by endotoxin in guinea pigs." *J Therm Biol*, 31(1-2), 220-228.

Ferguson, C. J., Wareing, M., Ward, D. T., Green, R., Smith, C. P., and Riccardi, D. (2001). "Cellular localization of divalent metal transporter DMT-1 in rat kidney." *American journal of physiology. Am J Physiol Renal Physiol*, 280(5), F803-14.

Ferring-appel, D., Hentze, M. W., and Galy, B. (2009). "Cell-autonomous and systemic context-dependent functions of iron regulatory protein 2 in mammalian iron metabolism." *Hematology*, 113(3), 679-687.

Fleck, C., Kretzschel, I., Sperschneider, T., and Appenroth, D. (2001). "Renal amino acid transport in immature and adult rats during chromate and cisplatinum-induced nephrotoxicity." *Amino Acids*, 20(2), 201-215.

Flo, T. H., Smith, K. D., Sato, S., Rodriguez, D. J., Holmes, M. A., Strong, R. K., Akira, S., and Aderem, A. (2004). "Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron." *Nature*, 432(7019), 917-21.

Frazer, D. M., Inglis, H. R., Wilkins, S. J., Millard, K. N., Steele, T. M., McLaren, G. D., McKie, A. T., Vulpe, C. D., and Anderson, G. J. (2004). "Delayed hepcidin response explains the lag period in iron absorption following a stimulus to increase erythropoiesis." *Gut*, 53(10), 1509-15.

Frazer, D. M., Wilkins, S. J., Becker, E. M., Vulpe, C. D., McKie, A. T., Trinder, D., and Anderson, G. J. (2002). "Hepcidin expression inversely correlates with the expression of duodenal iron transporters and iron absorption in rats." *Gastroenterology*, 123(3), 835-44.

Freudenberg, M. A., Tchaptchet, S., Keck, S., Huber, M., Schu, N., Beutler, B., and Galanos, C. (2008). "Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections : Benefits and hazards of LPS hypersensitivity." *Immunobiology*, 213, 193-203.

Friedl, A., Stoesz, S. P., Buckley, P., and Gould, M. N. (1999). "Neutrophil gelatinase-associated lipocalin in normal and neoplastic human tissues. Cell type-specific pattern of expression." *Histochem J*, 31(7), 433-41.

Galy, B., Ferring-Appel, D., Kaden, S., Gröne, H., and Hentze, M. W. (2008). "Iron regulatory proteins are essential for intestinal function and control key iron absorption molecules in the duodenum." *Cell Metab*, 7(1), 79-85.

Gnana-Prakasam, J. P., Martin, P. M., Smith, S. B., and Ganapathy, V. (2010). "Expression and function of iron-regulatory proteins in retina." *IUBMB life*, 62(5), 363-70.

Goetz, D. H., Holmes, M. A., Borregaard, N., Bluhm, M. E., Raymond, K. N., and Strong, R. K. (2002). "The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition." *Mol Cell*, 10(5), 1033-43.

Gomme, P. T., McCann, K. B., and Bertolini, J. (2005). "Transferrin: structure, function and potential therapeutic actions." *Drug Discov Today*, 10(4), 267-73.

Grzyb, J., Latowski, D., and Strzałka, K. (2006). "Lipocalins - a family portrait." *J Plant Physiol.*, 163(9), 895-915.

Guo, R., Wang, Y., Minto, A. W., Quigg, R. J., and Cunningham, P. N. (2004). "Acute renal failure in endotoxemia is dependent on caspase activation." *J Am Soc Nephrol*, 15(12), 3093-102.

Gwira, J. a., Wei, F., Ishibe, S., Ueland, J. M., Barasch, J., and Cantley, L. G. (2005). "Expression of neutrophil gelatinase-associated lipocalin regulates epithelial morphogenesis in vitro." *J Biol Chem*, 280(9), 7875-82.

Hale, J. S., and Fink, P. J. (2009). "Back to the thymus: peripheral T cells come home." *Immunol Cell Biol*, 87(1), 58-64.

Haley, P. (2003). "Species differences in the structure and function of the immune system." *Toxicology*, 188(1), 49-71.

Hanai, J., Mammoto, T., Seth, P., Mori, K., Karumanchi, S. A., Barasch, J., and Sukhatme, V. P. (2005). "Lipocalin 2 diminishes invasiveness and metastasis of Ras-transformed cells." *J Biol Chem*, 280(14), 13641-7.

Harju, K., Glumoff, V., and Hallman, M. (2001). "Ontogeny of Toll-like receptors Tlr2 and Tlr4 in mice." *Pediatr Res*, 49(1), 81-3.

Hauwel, M., Furon, E., Canova, C., Griffiths, M., Neal, J., and Gasque, P. (2005). "Innate (inherent) control of brain infection, brain inflammation and brain repair: the role of microglia, astrocytes, "protective" glial stem cells and stromal ependymal cells." *Brain research. Brain Res Brain Res Rev*, 48(2), 220-33.

Heemskerk, S., Pickkers, P., Bouw, M. P., Draisma, A., van der Hoeven, J. G., Peters, W. H., Smits, P., Russel, F. G., and Masereeuw, R. (2006). "Upregulation of renal inducible nitric oxide synthase during human endotoxemia and sepsis is associated with proximal tubule injury." *Clin J Am Soc Nephrol*, 1(4), 853-62.

Hentze, M. W., Muckenthaler, M. U., Galy, B., and Camaschella, C. (2010). "Two to tango: regulation of Mammalian iron metabolism." *Cell*, 142(1), 24-38.

Hentze, M. W., Muckenthaler, M. U., and Andrews, N. C. (2004). "Balancing acts: molecular control of mammalian iron metabolism." *Cell*, 117(3), 285-97.

Horster, M. (1977). "Nephron function and perinatal homeostasis." *Annales de recherches vétérinaires. Ann Rech Vet*, 8(4), 468-82.

Hower, V., Mendes, P., Torti, F. M., Laubenbacher, R., Akman, S., Shulaev, V., and Torti, S. V. (2009). "A general map of iron metabolism and tissue-specific subnetworks." *Mol Biosyst*, 5(5), 422-43.

Huang, X., Cho, S., and Spangrude, G. J. (2007). "Hematopoietic stem cells: generation and self-renewal." *Cell Death Differ*, 14(11), 1851-9.

Huebers, H. A., and Finch, C. A. (1987). "The physiology of transferrin and transferrin receptors." *Physiol Rev*, 67(2), 520-82.

Hurley, R. M., Nayyar, R. P., Goto, M., and Zeller, W. P. (1989). "Renal lesions in young rats induced by Salmonella enteritidis endotoxin." *Pediatr Nephrol*, 156-161.

Huston, J. M., Ochani, M., Rosas-Ballina, M., Liao, H., Ochani, K., Pavlov, V. a., Gallowitsch-Puerta, M., Ashok, M., Czura, C. J., Foxwell, B., Tracey, K. J., and Ulloa, L. (2006). "Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis." *J Exp Med*, 203(7), 1623-8.

Jarmi, T., and Agarwal, A. (2009). "Heme oxygenase and renal disease." *Curr Hypertens Rep*, 11(1), 56-62.

Jiang, W., Constante, M., and Santos, M. M. (2008). "Anemia upregulates lipocalin 2 in the liver and serum." *Blood Cells Mol Dis*, 41(2), 169-74.

Johnson, M. B., and Enns, C. A. (2004). "Diferric transferrin regulates transferrin receptor 2 protein stability." *Blood*, 104(13), 4287-93.

Kakhlon, O. (2002). "The labile iron pool: characterization, measurement, and participation in cellular processes." *Free Radic Biol Med*, 33(8), 1037-1046.

Kautz, L., Meynard, D., Monnier, A., Darnaud, V., Bouvet, R., Wang, R., Deng, C., Vaulont, S., Mosser, J., Coppin, H., and Roth, M. (2008). "Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver." *Blood*, 112(4), 1503-9.

Kehrer, J. P. (2009). "Lipocalin-2: pro- or anti-apoptotic?." *Cell Biol Toxicol*, (November 2008).

Kikuchi, G., Yoshida, T., and Noguchi, M. (2005). "Heme oxygenase and heme degradation." *Biochem Biophys Res Commun*, 338(1), 558-67.

Kjeldsen, L., Cowland, J. B., and Borregaard, N. (2000). "Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse." *Biochim Biophys Acta*, 1482(1-2), 272-83.

Kjeldsen, L., Johnsen, A. H., Sengeløv, H., and Borregaard, N. (1993). "Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase." *J Biol Chem*, 268(14), 10425-32.

Kozyraki, R., Fyfe, J., Verroust, P. J., Jacobsen, C., Dautry-Varsat, A., Gburek, J., Willnow, T. E., Christensen, E. I., and Moestrup, S. K. (2001). "Megalin-dependent

cubilin-mediated endocytosis is a major pathway for the apical uptake of transferrin in polarized epithelia." *Proc Natl Acad Sci U S A*, 98(22), 12491-6.

Kuchler, S., Graff, M. N., Gobaille, S., Vincendon, G., Roche, A. C., Delaunoy, J. P., Monsigny, M., and Zanetta, J. P. (1994). "Mannose dependent tightening of the rat ependymal cell barrier. In vivo and in vitro study using neoglycoproteins." *Neurochem Int*, 24(1), 43-55.

Kuwabara, T., Mori, K., Mukoyama, M., Kasahara, M., Yokoi, H., Saito, Y., Yoshioka, T., Ogawa, Y., Imamaki, H., Kusakabe, T., Ebihara, K., Omata, M., Satoh, N., Sugawara, A., Barasch, J., and Nakao, K. (2009). "Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons." *Kidney Int*, 75(3), 285-94.

Laflamme, N., and Rivest, S. (2001). "Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components." *FASEB J*, 15(1), 155-163.

Langenberg, C., Bagshaw, S. M., May, C. N., and Bellomo, R. (2008). "The histopathology of septic acute kidney injury: a systematic review." *Crit Care*, 12(2), R38.

Lee, H., Lee, E., Lee, K., Hong, S., Yoon, Y., and Kim, J. (2006). "Ectopic expression of neutrophil gelatinase-associated lipocalin suppresses the invasion and liver metastasis of colon cancer cells." *Int J Cancer*, 118(10), 2490-7.

Lee, P., Peng, H., Gelbart, T., Wang, L., and Beutler, E. (2005). "Regulation of hepcidin transcription by interleukin-1 and interleukin-6." *Proc Natl Acad Sci U S A*, 102(6), 1906-10.

Lee, S., Lee, J., Kim, S., Park, J., Lee, W., Mori, K., Kim, S., Kim, I. K., and Suk, K. (2007). "A dual role of lipocalin 2 in the apoptosis and deramification of activated microglia." *J Immunol*, 179(5), 3231-41.

Lee, S., Park, J., Lee, W., Kim, H., Park, H., Mori, K., and Suk, K. (2009). "Lipocalin-2 is an autocrine mediator of reactive astrocytosis." *J Neurosci*, 29(1), 234-49.

Leng, X., Ding, T., Lin, H., Wang, Y., Hu, L., Hu, J., Feig, B., Zhang, W., Pusztai, L., Symmans, W. F., Wu, Y., and Arlinghaus, R. B. (2009). "Inhibition of lipocalin 2 impairs breast tumorigenesis and metastasis." *Cancer Res*, 69(22), 8579-84.

Leng, X., Lin, H., Ding, T., Wang, Y., Wu, Y., Klumpp, S., Sun, T., Zhou, Y., Monaco, P., Belmont, J., Aderem, A., Akira, S., Strong, R., and Arlinghaus, R. (2008). "Lipocalin 2 is required for BCR-ABL-induced tumorigenesis." *Oncogene*, 27(47), 6110-9.

Levy, O., Zarember, K. A., Roy, R. M., Cywes, C., Godowski, P. J., and Wessels, M. R. (2004). "Selective impairment of TLR-mediated innate immunity in human

newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848." *J Immunol (Baltimore, Md. : 1950)*, 173(7), 4627-34.

Li, X., Zhang, C., Wang, H., Ji, Y., Wang, S., Zhao, L., Chen, X., and Xu, D. (2008). "Tumor necrosis factor alpha partially contributes to lipopolysaccharide-induced downregulation of CYP3A in fetal liver: its repression by a low dose LPS pretreatment." *Toxicol Lett*, 179(2), 71-7.

Lin, H., Monaco, G., Sun, T., Ling, X., Stephens, C., Xie, S., Belmont, J., and Arlinghaus, R. (2005). "Bcr-Abl-mediated suppression of normal hematopoiesis in leukemia." *Oncogene*, 24(20), 3246-56.

Liu, S., Gallo, D. J., Green, A. M., Williams, D. L., Gong, X., Shapiro, R. A., Gambotto, A. A., Humphris, E. L., Vodovotz, Y., and Billiar, T. R. (2002). "Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide." *Infect Immun*, 70(7), 3433-42.

Mandel, S., Amit, T., Bar-Am, O., and Youdim, M. B. (2007). "Iron dysregulation in Alzheimer's disease: multimodal brain permeable iron chelating drugs, possessing neuroprotective-neurorescue and amyloid precursor protein-processing regulatory activities as therapeutic agents." *Prog Neurobiol*, 82(6), 348-60.

Marques, F., Falcao, A. M., Sousa, J. C., Coppola, G., Geschwind, D., Sousa, N., Correia-Neves, M., and Palha, J. A. (2009). "Altered iron metabolism is part of the choroid plexus response to peripheral inflammation." *Endocrinology*, 150(6), 2822-8.

Marques, F., Rodrigues, A., Sousa, J. C., Coppola, G., Geschwind, D. H., Sousa, N., Correia-Neves, M., and Palha, J. A. (2008). "Lipocalin 2 is a choroid plexus acute-phase protein." *J Cereb Blood Flow Metab*, 28(3), 450-5.

Marques, F., Sousa, J. C., Coppola, G., Falcao, A. M., Rodrigues, A. J., Geschwind, D. H., Sousa, N., Correia-Neves, M., and Palha, J. a. (2009). "Kinetic profile of the transcriptome changes induced in the choroid plexus by peripheral inflammation." *J Cereb Blood Flow Metab*, 29(5), 921-32.

Marques, F., Sousa, J. C., Correia-Neves, M., Oliveira, P., Sousa, N., and Palha, J. a. (2007). "The choroid plexus response to peripheral inflammatory stimulus." *Neuroscience*, 144(2), 424-30.

Maródi, L. (2006). "Innate cellular immune responses in newborns." *Clin Immunol*, 118(2-3), 137-44.

McKie, A. T., Barrow, D., Latunde-Dada, G. O., Rolfs, A., Sager, G., Mudaly, E., Mudaly, M., Richardson, C., Barlow, D., Bomford, A., Peters, T. J., Raja, K. B., Shirali, S.,

Hediger, M. A., Farzaneh, F., and Simpson, R. J. (2001). "An iron-regulated ferric reductase associated with the absorption of dietary iron." *Science*, 291(5509), 1755-9.

McLean, E., Cogswell, M., Egli, I., Wojdyla, D., and de Benoist, B. (2009). "Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993-2005." *Public Health Nutr*, 12(4), 444-54.

Meheus, L. A., Fransen, L. M., Raymackers, J. G., Blockx, H. A., Van Beeumen, J. J., Van Bun, S. M., and Van De Voorde, A. (1993). "Identification by microsequencing of lipopolysaccharide-induced proteins secreted by mouse macrophages." *J Immunol*, 151(3), 1535-47.

Miharada, K., Hiroyama, T., Sudo, K., Danjo, I., Nagasawa, T., and Nakamura, Y. (2008). "Lipocalin 2-mediated growth suppression is evident in human erythroid and monocyte/macrophage lineage cells." *J Cell Physiol*, 215(2), 526-37.

Miharada, K., Hiroyama, T., Sudo, K., Nagasawa, T., and Nakamura, Y. (2005). “Lipocalin 2 functions as a negative regulator of red blood cell production in an autocrine fashion”. *FASEB J*, 19(13), 1881-3.

Mikkola, H. K., and Orkin, S. H. (2006). "The journey of developing hematopoietic stem cells." *Development*, 133(19), 3733-44.

Millard, K. N., Frazer, D. M., Wilkins, S. J., and Anderson, G. J. (2004). "Changes in the expression of intestinal iron transport and hepatic regulatory molecules explain the enhanced iron absorption associated with pregnancy in the rat." *Gut*, 53(5), 655-60.

Mishra, J., Dent, C., Tarabishi, R., Mitsnefes, M. M., Ma, Q., Kelly, C., Ruff, S. M., Zahedi, K., Shao, M., Bean, J., Mori, K., Barasch, J., and Devarajan, P. (2005). "Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery." *Lancet*, 365(9466), 1231-8.

Mishra, J., Ma, Q., Kelly, C., Mitsnefes, M., Mori, K., Barasch, J., and Devarajan, P. (2006). "Kidney NGAL is a novel early marker of acute injury following transplantation." *Pediatr Nephrol*, 21(6), 856-63.

Mishra, J., Ma, Q., Prada, A., Mitsnefes, M., Zahedi, K., Yang, J., Barasch, J., and Devarajan, P. (2003). "Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury." *J Am Soc Nephrol*, 14(10), 2534-43.

Mishra, J., Mori, K., Ma, Q., Kelly, C., Barasch, J., and Devarajan, P. (2004). "Neutrophil gelatinase-associated lipocalin: a novel early urinary biomarker for cisplatin nephrotoxicity." *Am J Nephrol*, 24(3), 307-15.

Mishra, J., Mori, K., Ma, Q., Kelly, C., Yang, J., Mitsnefes, M., Barasch, J., and Devarajan, P. (2004). "Amelioration of ischemic acute renal injury by neutrophil gelatinase-associated lipocalin." *J Am Soc Nephrol*, 15(12), 3073-82.

Mitsnefes, M. M., Kathman, T. S., Mishra, J., Kartal, J., Khoury, P. R., Nickolas, T. L., Barasch, J., and Devarajan, P. (2007). "Serum neutrophil gelatinase-associated lipocalin as a marker of renal function in children with chronic kidney disease." *Pediatr Nephrol*, 22(1), 101-8.

Mori, K., Lee, H. T., Rapoport, D., Drexler, I. R., Foster, K., Yang, J., Schmidt-Ott, K. M., Chen, X., Li, J. Y., Weiss, S., Mishra, J., Cheema, F. H., Markowitz, G., Suganami, T., Sawai, K., Mukoyama, M., Kunis, C., D'Agati, V., Devarajan, P., and Barasch, J. (2005). "Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury." *J Clin Invest*, 115(3), 610-21.

Mori, K., and Nakao, K. (2007). "Neutrophil gelatinase-associated lipocalin as the real-time indicator of active kidney damage." *Kidney Int*, 71(10), 967-70.

Muckenthaler, M. U., Galy, B., and Hentze, M. W. (2008). "Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network." *Annu Rev Nutr*, 28, 197-213.

Muñoz, M., Villar, I., and García-Erce, J. A. (2009). "An update on iron physiology." *World J Gastroenterol*, 15(37), 4617-26.

Nadadur, S. S., Srirama, K., and Mudipalli, A. (2008). "Iron transport & homeostasis mechanisms: their role in health & disease." *Indian J Med Res*, 128(4), 533-44.

Nadeau, S., and Rivest, S. (1999). "Regulation of the gene encoding tumor necrosis factor alpha (TNF-alpha) in the rat brain and pituitary in response in different models of systemic immune challenge." *J Neuropathol Exp Neurol*, 58(1), 61-77.

Neilands, J. B. (1995). "Siderophores: structure and function of microbial iron transport compounds." *J Biol Chem*, 270(45), 26723-6.

Nelson, N. (1999). "Metal ion transporters and homeostasis." *EMBO J*, 18(16), 4361-71.

Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M., Ganz, T., and Kaplan, J. (2004). "Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization." *Science*, 306(5704), 2090-3.

Nemeth, E., and Ganz, T. (2009). "The role of hepcidin in iron metabolism." *Acta Haematol*, 122(2-3), 78-86.

Nicolas, G., Bennoun, M., Devaux, I., Beaumont, C., Grandchamp, B., Kahn, a., and Vaulont, S. (2001). "Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice." *Proc Natl Acad Sci U S A*, 98(15), 8780-5.

Nicolas, G., Bennoun, M., Porteu, A., Mativet, S., Beaumont, C., Grandchamp, B., Sirito, M., Sawadogo, M., Kahn, A., and Vaulont, S. (2002). "Severe iron deficiency anemia in transgenic mice expressing liver hepcidin." *Proc Natl Acad Sci U S A*, 99(7), 4596-601.

Nielsen, B. S., Borregaard, N., Bundgaard, J. R., Timshel, S., Sehested, M., and Kjeldsen, L. (1996). "Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases." *Gut*, 38(3), 414-20.

Ning, H., Wang, H., Zhao, L., Zhang, C., Li, X., Chen, Y., and Xu, D. (2008). "Maternally-administered lipopolysaccharide (LPS) increases tumor necrosis factor alpha in fetal liver and fetal brain : Its suppression by low-dose LPS pretreatment." *Toxicol Lett*, 176, 13-19.

Nussler, a. K., Wittel, U. a., Nussler, N. C., and Beger, H. G. (1999). "Leukocytes, the Janus cells in inflammatory disease." *Langenbecks Arch Surg*, 384(2), 222-32.

Ojaniemi, M., Liljeroos, M., Harju, K., Sormunen, R., Vuolteenaho, R., and Hallman, M. (2006). "TLR-2 is upregulated and mobilized to the hepatocyte plasma membrane in the space of Disse and to the Kupffer cells TLR-4 dependently during acute endotoxemia in mice." *Immunology Lett*, 102(2), 158-68.

Orelio, C., Peeters, M., Haak, E., van Der Horn, K., and Dzierzak, E. (2009). "Interleukin-1 regulates hematopoietic progenitor and stem cells in the midgestation mouse fetal liver." *Haematologica*, 94(4), 462-9.

Orkin, S. H., and Zon, L. I. (2008). "Hematopoiesis: an evolving paradigm for stem cell biology." *Cell*, 132(4), 631-44.

Park, C. H., Valore, E. V., Waring, A. J., and Ganz, T. (2001). "Hepcidin, a urinary antimicrobial peptide synthesized in the liver." *J Biol Chem*, 276(11), 7806-10.

Parker, L. C., Prince, L. R., and Sabroe, I. (2007). "Translational mini-review series on Toll-like receptors: networks regulated by Toll-like receptors mediate innate and adaptive immunity." *Clin Exp Immunol*, 147(2), 199-207.

Pearse, G. (2006). "Normal structure, function and histology of the thymus." *Toxicol Pathol*, 34(5), 504-14.

Pigeon, C., Ilyin, G., Courselaud, B., Leroyer, P., Turlin, B., Brissot, P., and Loréal, O. (2001). "A new mouse liver-specific gene, encoding a protein homologous to human

antimicrobial peptide hepcidin, is overexpressed during iron overload." *J Biol Chem*, 276(11), 7811-9.

Playford, R. J., Belo, A., Poulsom, R., Fitzgerald, A. J., Harris, K., Pawluczyk, I., Ryon, J., Darby, T., Nilsen-Hamilton, M., Ghosh, S., and Marchbank, T. (2006). "Effects of mouse and human lipocalin homologues 24p3/lcn2 and neutrophil gelatinase-associated lipocalin on gastrointestinal mucosal integrity and repair." *Gastroenterology*, 131(3), 809-17.

Quan, N., Stern, E. L., Whiteside, M. B., and Herkenham, M. (1999). "Induction of pro-inflammatory cytokine mRNAs in the brain after peripheral injection of subseptic doses of lipopolysaccharide in the rat." *J Neuroimmunol*, 93(1-2), 72-80.

Rached, E., Hoffmann, D., Blumbach, K., Weber, K., Dekant, W., and Mally, A. (2008). "Evaluation of putative biomarkers of nephrotoxicity after exposure to ochratoxin a in vivo and in vitro." *Toxicol Sci*, 103(2), 371-81.

Ramaiah, S. K., and Jaeschke, H. (2007). "Role of neutrophils in the pathogenesis of acute inflammatory liver injury." *Toxicol Pathol*, 35(6), 757-66.

Ramesh, G., Zhang, B., Uematsu, S., Akira, S., and Reeves, W. B. (2007). "Endotoxin and cisplatin synergistically induce renal dysfunction and cytokine production in mice." *Am J Physiol Renal Physiol*, 293(1), F325-32.

Ramey, G., Deschemin, J., Durel, B., Canonne-hergaux, F., Nicolas, G., Vaulont, S., Cochin, I., Descartes, U. P., Umr, C., and Chimie, I. D. (2010). "Hepcidin targets ferroportin for degradation in hepatocytes." *Haematologica,* 95(3), 501-504.

Randolf, A., Wildmann, J., Besedovsky, H. O., and Jessop, D. S. (2009). "Brain , Behavior , and Immunity Re-exposure to endotoxin induces differential cytokine gene expression in the rat hypothalamus and spleen." *Brain Behav Immun*, 23(6), 776-783.

Redzic, Z. B., and Segal, M. B. (2004). "The structure of the choroid plexus and the physiology of the choroid plexus epithelium." *Adv Drug Deliv Rev*, 56(12), 1695-716.

Richardson, D. R. (2005). "24p3 and its receptor: dawn of a new iron age?." *Cell*, 123(7), 1175-7.

Robertson, S. a., Care, A. S., and Skinner, R. J. (2007). "Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in mice." *Biol Reprod*, 76(5), 738-48.

Robertson, S. A., Skinner, R. J., and Care, A. S. (2006). “Essential role for IL-10 in resistance to lipopolysaccharide-induced preterm labor in mice.” *J Immunol*, 177(7), 4888-96.

Roudkenar, M. H., Halabian, R., Roushandeh, A. M., Nourani, M. R., Masroori, N., Ebrahimi, M., Nikogoftar, M., Rouhbakhsh, M., Bahmani, P., Najafabadi, A. J., and Shokrgozar, M. A. (2009). "Lipocalin 2 regulation by thermal stresses: Protective role of Lcn2/NGAL against cold and heat stresses." *Exp Cell Res,* 315(18), 3140-51

Sasaki, K., and Sonoda, Y. (2000). "Histometrical and three-dimensional analyses of liver hematopoiesis in the mouse embryo." *Arch Histol Cytol*, 63(2), 137-46.

Savino, W. (2000). "Neuroendocrine Control of Thymus Physiology." *Endocr Rev*, 21(4), 412-443.

Savino, W., Dardenne, M., Velloso, L. A., and Dayse Silva-Barbosa, S. (2007). "The thymus is a common target in malnutrition and infection." *Br J Nutr*, 98 Suppl 1, S11-6.

Sawazaki, K., Yasuda, T., Nadano, D., Iida, R., and Kishi, K. (1992). "Transferrin (TF) typing from semen stains using isoelectric focusing and immunoblotting: correlation of TF types among blood, semen, urine, and vaginal secretion." *J Forensic Sci*, 37(6), 1514-24.

Schmidt-Ott, K. M., Mori, K., Kalandadze, A., Li, J., Paragas, N., Nicholas, T., Devarajan, P., and Barasch, J. (2006). "Neutrophil gelatinase-associated lipocalin-mediated iron traffic in kidney epithelia." *Curr Opin Nephrol Hypertens*, 15(4), 442-9.

Schmidt-Ott, K. M., Mori, K., Li, J. Y., Kalandadze, A., Cohen, D. J., Devarajan, P., and Barasch, J. (2007). "Dual action of neutrophil gelatinase-associated lipocalin." *J Am Soc Nephrol*, 18(2), 407-13.

Schrier, R. W., and Wang, W. (2004). "Acute Renal Failure and Sepsis." *Shock*, 159-169.

Shaw, L., Johnson, P. a., and Kimber, S. J. (2009). "Gene expression profiling of the developing mouse kidney and embryo." *In Vitro Cell Dev Biol Anim*., 46(2), 155-65

Skipor, J., and Thiery, J. (2008). "The choroid plexus--cerebrospinal fluid system: undervaluated pathway of neuroendocrine signaling into the brain." *Acta Neurobiol Exp (Wars)*, 68(3), 414-28.

Smith, C. P., and Thévenod, F. (2009). "Iron transport and the kidney." *Biochim Biophys Acta*, 1790(7), 724-30.

Smolinski, A. T., and Pestka, J. J. (2005). "Comparative effects of the herbal constituent parthenolide (Feverfew) on lipopolysaccharide-induced inflammatory gene expression in murine spleen and liver." *J Inflamm (Lond)*, 2, 6.

Solhaug, M. J., Bolger, P. M., and Jose, P. A. (2004). "The developing kidney and environmental toxins." *Pediatrics*, 113(4 Suppl), 1084-91.

Sommer, G., Weise, S., Kralisch, S., Lossner, U., Bluher, M., Stumvoll, M., and Fasshauer, M. (2009). "Lipocalin-2 is induced by interleukin-1beta in murine adipocytes in vitro." *J Cell Biochem*, 106(1), 103-8.

Sousa, J. C., Cardoso, I., Marques, F., Saraiva, M. J., and Palha, J. A. (2007). "Transthyretin and Alzheimer's disease: where in the brain?." *Neurobiol Aging*, 28(5), 713-8.

Speake, T., Whitwell, C., Kajita, H., Majid, a., and Brown, P. D. (2001). "Mechanisms of CSF secretion by the choroid plexus." *Microsc Res Tech*, 52(1), 49-59.

Sunil, V. R., Patel, K. J., Nilsen-Hamilton, M., Heck, D. E., Laskin, J. D., and Laskin, D. L. (2007). "Acute endotoxemia is associated with upregulation of lipocalin 24p3/Lcn2 in lung and liver." *Exp Mol Pathol*, 83(2), 177-87.

Sutherland, J., Goldberg, G., Hammett, M., Uldrich, A., Berzins, S., Heng, T., Blazar, B., Millar, J., Malin, M., Chidgey, A., and others. (2005). "Activation of thymic regeneration in mice and humans following androgen blockade." *J Immunol*, Am Assoc Immnol, 175(4), 2741.

Suzuki, M. (2009). "Children's toxicology from bench to bed - Drug-induced Renal Injury (4): Effects of nephrotoxic compounds on fetal and developing kidney." *J Toxicol Sci*, 34 Suppl 2, SP267-SP271.

Tada, T., Widayati, D. T., and Fukuta, K. (2006). "Morphological study of the transition of haematopoietic sites in the developing mouse during the peri-natal period." *Anat Histol Embryol*, 35(4), 235-40.

Tanno, T., Bhanu, N. V., Oneal, P. A., Goh, S., Staker, P., Lee, Y. T., Moroney, J. W., Reed, C. H., Luban, N. L., Wang, R., Eling, T. E., Childs, R., Ganz, T., Leitman, S. F., Fucharoen, S., and Miller, J. L. (2007). "High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin." *Nat Med*, 13(9), 1096-101.

Tilg, H., Dinarello, C. A., and Mier, J. W. (1997). "IL-6 and APPs: anti-inflammatory and immunosuppressive mediators." *Immunol Today*, 18(9), 428-32.

Tong, Z., Wu, X., Ovcharenko, D., Zhu, J., Chen, C., and Kehrer, J. P. (2005). "Neutrophil gelatinase-associated lipocalin as a survival factor." *Biochem J*, 391(Pt 2), 441-8.

Tong, Z., Wu, X., and Kehrer, J. P. (2003). "Increased expression of the lipocalin 24p3 as an apoptotic mechanism for MK886." *Biochem J*, 210, 203-210.

Torti, F. M., Updated, S. V., Cells, R., Expression, G., Articles, R., Society, A., Dc, W., and Society, T. A. (2002). "Regulation of ferritin genes and protein." *Blood*, 99(10), 3505-3516.

Tracey, K. J. (2007). "Physiology and immunology of the cholinergic antiinflammatory pathway." *J Clin Invest*, 117(2), 289-96.

Trachtman, H., Christen, E., Cnaan, A., Patrick, J., Mai, V., Mishra, J., Jain, A., Bullington, N., and Devarajan, P. (2006). "Urinary neutrophil gelatinase-associated lipocalcin in D+HUS: a novel marker of renal injury." *Pediatr Nephrol*, 21(7), 989-94.

Tracz, M. J., Juncos, J. P., Grande, J. P., Croatt, a. J., Ackerman, a. W., Rajagopalan, G., Knutson, K. L., Badley, a. D., Griffin, M. D., Alam, J., and Nath, K. a. (2007). "Renal Hemodynamic, Inflammatory, and Apoptotic Responses to Lipopolysaccharide in HO-1-/- Mice." *Am J Pathol*, 170(6), 1820-1830.

Trinder, D., Fox, C., Vautier, G., and Olynyk, J. K. (2002). "Molecular pathogenesis of iron overload.." *Gut*, 51(2), 290-5.

Veuthey, T. V., and Roque, M. E. (2009). "Immunolocalization of Ferroportin in Healthy and Anemic Mice." *J Histochem Cytochem*, 57(1), 9-16.

Veuthey, T., D'Anna, M. C., and Roque, M. E. (2008). "Role of the kidney in iron homeostasis: renal expression of Prohepcidin, Ferroportin, and DMT1 in anemic mice." *Am J Physiol Renal Physiol*, 295(4), F1213-21.

Viatte, L., Gröne, H., Hentze, M. W., and Galy, B. (2009). "In vivo role(s) of the iron regulatory proteins (IRP) 1 and 2 in aseptic local inflammation." *J Mol Med*, 87(9), 913-21.

Viatte, L., and Vaulont, S. (2009). "Hepcidin, the iron watcher." *Biochimie*, 91(10), 1223-8.

Vulpe, C. D., Kuo, Y. M., Murphy, T. L., Cowley, L., Askwith, C., Libina, N., Gitschier, J., and Anderson, G. J. (1999). "Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse." *Nat Genet*, 21(2), 195-9.

Wagener, G., Jan, M., Kim, M., Mori, K., Barasch, J. M., Sladen, R. N., and Lee, H. T. (2006). "Association between increases in urinary neutrophil gelatinase-associated lipocalin and acute renal dysfunction after adult cardiac surgery." *Anesthesiology*, 105(3), 485-91.

Walker, P. R., Smith, C., Youdale, T., Leblanc, J., Whitfield, J. F., and Sikorska, M. (1991). "Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes." *Cancer Res*, 51(4), 1078-85.

Wan, L., Bagshaw, S. M., Langenberg, C., Saotome, T., May, C., and Bellomo, R. (2008). "Pathophysiology of septic acute kidney injury: what do we really know?." *Crit Care Med*, 36(4 Suppl), S198-203.

Wang, E., Snyder, R. D., Fielden, M. R., Smith, R. J., and Gu, Y. (2008). "Validation of putative genomic biomarkers of nephrotoxicity in rats." *Toxicology*, 246, 91-100.

Wang, W., Faubel, S., Ljubanovic, D., Mitra, A., Falk, S. a., Kim, J., Tao, Y., Soloviev, A., Reznikov, L. L., Dinarello, C. a., Schrier, R. W., and Edelstein, C. L. (2005). "Endotoxemic acute renal failure is attenuated in caspase-1-deficient mice." *Am J Physiol Renal Physiol*, 288(5), F997-1004.

Wang, X., Rousset, C. I., Hagberg, H., and Mallard, C. (2006). "Lipopolysaccharide-induced inflammation and perinatal brain injury." *Fetal Neonatal Med*, 11(5), 343-53.

Wareing, M., Ferguson, C. J., Delannoy, M., Cox, A. G., McMahon, R. F., Green, R., Riccardi, D., and Smith, C. P. (2003). "Altered dietary iron intake is a strong modulator of renal DMT1 expression." *Am J Physiol Renal Physiol*, 285(6), F1050-9.

Wareing, M., Ferguson, C. J., Green, R., Riccardi, D., and Smith, C. P. (2000). "In vivo characterization of renal iron transport in the anaesthetized rat." *J Physiol*, 524 Pt 2, 581-6.

Wheeler, D. S., Devarajan, P., Ma, Q., Harmon, K., Monaco, M., Cvijanovich, N., and Wong, H. R. (2008). "Serum neutrophil gelatinase-associated lipocalin (NGAL) as a marker of acute kidney injury in critically ill children with septic shock." *Crit Care Med*, 36(4), 1297-303.

Wit, D. D., Tonon, S., Goriely, S., Goldman, M., and Willems, F. (2003). "Impaired responses to toll-like receptor 4 and toll-like receptor 3 ligands in human cord blood." *J Autoimmun*, 21, 277-281.

Wolber, F. M., Leonard, E., Michael, S., Orschell-Traycoff, C. M., Yoder, M. C., and Srour, E. F. (2002). "Roles of spleen and liver in development of the murine hematopoietic system." *Exp Hematol*, 30(9), 1010-9.

Wolfs, T. G., Buurman, W. A., van Schadewijk, A., de Vries, B., Daemen, M. A., Hiemstra, P. S., and van 't Veer, C. (2002). "In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation." *J Immunol*, 168(3), 1286-93.

Wrighting, D. M., and Andrews, N. C. (2006). "Interleukin-6 induces hepcidin expression through STAT3." *Blood*, 108(9), 3204-9.

Wu, L., Tiwari, M. M., Messer, K. J., Holthoff, J. H., Gokden, N., Brock, R. W., and Mayeux, P. R. (2007). "Peritubular capillary dysfunction and renal tubular epithelial cell stress following lipopolysaccharide administration in mice." *Am J Physiol Renal Physiol*, 292(1), F261-8.

Xia, Y., Yamagata, K., and Krukoff, T. L. (2006). "Differential expression of the CD14/TLR4 complex and inflammatory signaling molecules following i.c.v. administration of LPS." *Brain Res*, 1095(1), 85-95.

Xu, D., Wang, H., Zhao, L., Ning, H., Chen, Y., and Zhang, C. (2007). "Effects of low-dose lipopolysaccharide (LPS) pretreatment on LPS-induced intra-uterine fetal death and preterm labor." *Toxicology*, 234(3), 167-75.

Yang, J., Goetz, D., Li, J. Y., Wang, W., Mori, K., Setlik, D., Du, T., Erdjument-Bromage, H., Tempst, P., Strong, R., and Barasch, J. (2002). "An iron delivery pathway mediated by a lipocalin." *Mol cell*, 10(5), 1045-56.

Yang, J., Mori, K., Li, J. Y., and Barasch, J. (2003). "Iron, lipocalin, and kidney epithelia." *Am J Physiol Renal Physiol*, 285(1), F9-18.

Yeh, K. Y., Yeh, M., Watkins, J. A., Rodriguez-Paris, J., and Glass, J. (2000). "Dietary iron induces rapid changes in rat intestinal divalent metal transporter expression." *Am J Physiol Gastrointest Liver Physiol*, 279(5), G1070-9.

Yura, R. E., Bradley, S. G., Ramesh, G., Reeves, W. B., and Bond, J. S. (2009). "Meprin A metalloproteases enhance renal damage and bladder inflammation after LPS challenge." *Am J Physiol Renal Physiol*, 296(1), F135-44.

Zager, R. A., Johnson, A. C., Naito, M., Lund, S. R., Kim, N., and Bomsztyk, K. (2008). "Growth and development alter susceptibility to acute renal injury." *Kidney Int*, 74(5), 674-8.

Zager, R. a., Johnson, A. C., Lund, S., and Hanson, S. (2006). "Acute renal failure: determinants and characteristics of the injury-induced hyperinflammatory response." *Am J Physiol Renal Physiol*, 291(3), F546-56.

Zhang, C., Walker, L. M., and Mayeux, P. R. (2000). "Role of nitric oxide in lipopolysaccharide-induced oxidant stress in the rat kidney." *Biochem Pharmacol*, 59(2), 203-9.

Zhang, D., Meyron-holtz, E., and Rouault, T. A. (2007). "Transferrin iron delivery and the role of iron regulatory proteins." *Am. Soc. Nephrol.*, 18, 401-406.

Zhang, J., Wu, Y., Zhang, Y., Leroith, D., Bernlohr, D. a., and Chen, X. (2008). "The role of lipocalin 2 in the regulation of inflammation in adipocytes and macrophages." *Mol Endocrinol*, 22(6), 1416-26.